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BRCA1, a hereditary breast and ovarian specific tumor suppressor, ensures genomic integrity through its control of transcription and repair of damaged DNA. Considerable evidence implicates DNA damage-induced site-specific phosphorylation in the modulation of its biological activity. However, it is not presently clear whether and how the transcription and DNA repair activities of BRCA1 are modulated in response to DNA damage signals. We have engineered and refined a unique combination of biochemical and genetic tools to address this issue. First, we have developed a biochemical means by which to resolve BRCA1-containing complexes involved in transcription from those involved in DNA double-strand break repair. This should render it feasible to identify DNA damage-induced site-specific phosphorylation events with potential functional relevance to the role of BRCA1 in these two processes. Second, we have established fibroblast cultures from brca1-deficient mouse embryos and developed BRCA1-dependent transcription and repair assays based on the use of these cells. This system will expedite the facile and efficient analysis of the effects of targeted BRCA1 mutations at identified or predicted sites of phosphorylation on its transcription and DNA repair activities. Collectively, these studies should illuminate the molecular basis for the caretaker properties of BRCA1.

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INTRODUCTION

BRCA1, a hereditary breast- and ovarian-specific tumor suppressor, functions in the global maintenance of genome stability, and has been implicated in both transcription and DNA double-strand break repair processes. Considerable evidence implicates DNA-damage-induced site-specific phosphorylation of BRCA1 as a critical regulator of its caretaker properties. However, it is not presently known whether and how the transcription and/or DNA repair activities of BRCA1 are specifically modulated in response to DNA damage. We hypothesized that DNA damage-induced site-specific phosphorylation of BRCA1 regulates its transcription and/or DNA double-strand break repair activities. To provide support for this hypothesis, we proposed first to identify ionizing radiation-induced site-specifically phosphorylated residues on BRCA1 in complex with transcription or DNA double-strand break repair activities, and second, to determine the functional consequence of ionizing radiation-induced site-specific phosphorylation on the transcription and DNA double-strand break repair activities of BRCA1. Toward this objective, our plan has been first to biochemically purify from human cells, both prior to and following irradiation, distinct BRCA1-containing multiprotein complexes corresponding to the RNA polymerase II holoenzyme and the Rad50/Mre11/NBS1 DNA double-strand break (DSB) repair complex, and second to effect direct comparative analyses of wild type BRCA1 and mutant derivatives bearing substitutions at ionizing radiation-targeted residues for their respective abilities to function in BRCA1-dependent transcription and DNA double-strand break repair assays *in vivo*.

BODY

Technical Objective 1. To identify ionizing irradiation (IR)-induced sitespecifically phosphorylated residues on BRCA1 in complex with either the RNA polymerase II holoenzyme or the Rad50/Mre11/NBS1 DNA double-strand break repair complex.

Task 1: ***Months 1-9:*** To purify distinct BRCA1-containing complexes corresponding to the RNA polymerase II holoenzyme and the Rad50/Mre11/NBS1 DNA double-strand break repair complex both prior to and following IR.

We have achieved the biochemical purification of distinct BRCA1-containing multiprotein complexes implicated in transcription and DNA repair (Please Refer to Appendix 1 – Manuscript Preprint). We are now in the process of establishing the function of these transcription and DNA repair complexes in vitro using reconstituted transcription and DNA repair assays.

During the course of our biochemical fractionation studies, we discovered that a multiprotein complex comprised of BRCA1 in association with transcription activities included an established transcriptional co-repressor protein, N-CoR (nuclear receptor co-repressor). N-CoR is a 270 kDa protein that was originally identified based upon its ability to bind to and mediate transcriptional repression by unliganded class II nuclear receptors, including the thyroid hormone receptor. Interestingly, N-CoR also mediates

the anti-estrogenic effects of tamoxifen, a selective modulator of the estrogen receptor α (ERα) and a chemotherapeutic agent used to treat breast cancer. Tamoxifen-bound ERα is repressed by virtue of its direct interaction with N-CoR, which resides in stable association with a multiprotein histone deacetylase (HDAC) transcriptional repression complex.

The identification of N-CoR in stable complex with BRCA1 suggested the possibility of a functional link between BRCA1 and nuclear hormone receptors, including the ERα. Such a link could have important implications with respect to the role of BRCA1 as a breast and ovarian-specific tumor suppressor protein. BRCA1 ensures global genome stability through its dual participation in DNA double-strand break repair and transcriptional regulation of DNA damage-inducible genes that function in cell cycle checkpoint control. Because the DNA damage-induced signaling pathways that converge on BRCA1 are conserved in most cell types, BRCA1 is likely to function ubiquitously in the maintenance of genome integrity. Nonetheless, germline inactivation of BRCA1 leads principally to cancers of the breast and ovary, and the underlying basis for its tissue-restricted tumor suppressor function thus remains poorly defined. In pursuing the potential functional link between BRCA1 and ERα, we discovered a novel function for BRCA1 in suppressing the ligand-independent transcriptional activity of ERα. Importantly, we documented that clinically validated BRCA1 missense mutations abrogate this repression activity, thereby suggesting that its ERα-specific repression function is important for the biological activity of BRCA1 in breast and ovarian tumor suppression. In human breast cancer cells, we observed an association between BRCA1 and ERα at endogenous estrogen-responsive gene promoters before, but not after, estrogen stimulation. Furthermore, we demonstrated that attenuation of BRCA1 expression in estrogen-dependent human ovarian cancer cells could be correlated with increases in both the estrogen-independent transcription of ERα-regulated genes and estrogen-independent cellular proliferation. These are extremely novel and significant findings because they suggest a possible mechanism by which functional inactivation of BRCA1 could promote tumorigenesis through inappropriate hormonal regulation of mammary and ovarian epithelial cell proliferation. These studies have since been published in manuscript form (Please refer to Appendix 2 – Manuscript Reprint). Based on these extremely important findings, we are planning to seek extramural funding to pursue studies concerning the modulation of estrogen receptor activity by BRCA1.

Task 2: ***Months 3-18:*** To identify IR-induced site-specifically phosphorylated residues on BRCA1 present in purified RNA polymerase II holoenzyme and Rad50/Mre11/NBS1 DNA double-strand break repair complexes by both mass spectrometric analyses and immunoblot analyses using phosphopeptide-specific antibodies.

We are currently engaged in efforts to identify IR-induced site-specifically phosphorylated residues on BRCA1 present in purified RNA polymerase II holoenzyme and Rad50/Mre11/NBS1 DNA double-strand break repair complexes by mass spectrometric analyses.

Technical Objective 2. To determine the functional consequence of individual IR-induced site-specific phosphorylation events on the transcription and DNA double-strand break repair activities of BRCA1.

Task 1: ***Months 6-24:*** To determine the effects of targeted mutations at identified (or predicted) sites of IR-induced phosphorylation within BRCA1 on its ability to activate transcription following its ectopic expression in brca1-deficient cells.

These studies will be implemented once we identify IR-induced site-specifically phosphorylated residues on BRCA1 – studies in which we are presently engaged.

Task 2: ***Months 9-36:*** To determine the effects of targeted mutations at identified (or predicted) sites of IR-induced phosphorylation within BRCA1 on its ability to effect DNA double-strand break repair by homologous recombination and non-homologous end-joining following its ectopic expression in Brca1-deficient cells.

These studies will be implemented once we identify IR-induced site-specifically phosphorylated residues on BRCA1 – studies in which we are presently engaged.

KEY RESEARCH ACCOMPLISHMENTS

- Biochemical resolution of distinct BRCA1-containing multiprotein complexes implicated in transcription and DNA repair.
- Novel discovery that BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor.

REPORTABLE OUTCOMES

Manuscripts:

1. **Boyer, T.G.** and Lee W-H: Biochemical Resolution of Distinct BRCA1-Containing Multiprotein Complexes Implicated in Transcription and DNA Repair. *Submitted* (2000). ***Please Refer to Appendix 1.***
2. Zheng, L., Annab, L.A., Afshari, C.A., Lee, W.-H., and **Boyer, T.G.** (2001) BRCA1 Mediates Ligand-Independent Transcriptional Repression of the Estrogen Receptor. *Proc. Natl. Acad. Sci. U.S.A* **98**: 9587-9592. ***Please Refer to Appendix 2.***

Reviews:

1. Lee, W.-H. and **Boyer, T.G.** (2001). BRCA1 and BRCA2 in breast cancer. *The Lancet* (Supplement), **358:** S5. ***Please Refer to Appendix 3.***

2. **Boyer, T.G.** and Lee, W.-H. (2002). Breast Cancer Susceptibility Genes. *Science & Medicine* **8**: 138-149. ***Please Refer to Appendix 4.***

Awards:

1. Career Development Award DAMD17-02-1-0584, U.S. Army Department of Defense, BCRP. 2002

CONCLUSIONS

We have succeeded in the biochemical resolution of distinct BRCA1-containing multiprotein complexes implicated in transcription and DNA repair. This should now render it feasible to identify by a mass spectrometric-based approach DNA damage-induced site-specific phosphorylation events with potential functional relevance to the role of BRCA1 in these two processes. Over the next two years, we will exploit BRCA1-dependent transcription and repair-based assays in *Brca1-/-* mouse embryo fibroblast cells to analyze of the effects of targeted BRCA1 mutations at identified sites of phosphorylation on its transcription and DNA repair activities. Collectively, these studies should illuminate the molecular basis for the caretaker properties of BRCA1.

We have identified a novel function for BRCA1 in suppressing the ligand-independent transcriptional activity of the estrogen receptor α (ERα), a principal determinant of the growth and differentiation of breasts and ovaries. Importantly, we documented that clinically validated BRCA1 missense mutations abrogate this repression activity, thereby suggesting that its ERα-specific repression function is important for the biological activity of BRCA1 in breast and ovarian tumor suppression. Our results thus reveal BRCA1 to be a ligand-reversible barrier to transcriptional activation by unliganded ERα, and suggest a possible mechanism by which functional inactivation of BRCA1 could promote tumorigenesis through inappropriate hormonal regulation of breast epithelial cell proliferation. These studies offer possible insight into the tissue-specific tumor suppressor function of BRCA1 and could suggest defined molecular targets for future intevention in breast cancer.

Biochemical Resolution of Distinct BRCA1-Containing Multiprotein Complexes Implicated in Transcription and DNA Repair

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Running Title: BRCA1 complexes implicated in transcription and DNA repair

BRCA1, a hereditary breast- and ovarian-specific tumor suppressor, functions in the maintenance of genome integrity and has been implicated in a diverse range of cellular processes including transcription regulation and DNA repair. However, the physical and functional relationship between BRCA1-containing activities involved in these processes remains to be fully deciphered. Here, we report the biochemical resolution of distinct multiprotein complexes comprised of BRCA1 in association with transcription and DNA repair activities. One complex, consisting of BRCA1, NBS1, Rad50, RNA polymerase II, and RNA polymerase II Mediator proteins could be resolved from a second complex comprised of BRCA1, NBS1, Rad50, Mre11, and additional polypeptides. These findings provide biochemical evidence for stable and distinct BRCA1-containing complexes with potential roles in transcription and DNA repair and, furthermore, provide evidence for an interaction of NBS1 and Rad50 with the RNA polymerase II holoenzyme. The presence of BRCA1, NBS1, and Rad50 in distinct complexes raises the possibility that these proteins represent a common core through which transcription and repair activities may be physically and functionally linked within the cell.

INTRODUCTION

Hereditary predisposition to early onset breast and ovarian cancer derives principally from germ-line mutations in either of two **BR**east **CA**ncer susceptibility genes, ***BRCA1*** and ***BRCA2*** (1,2). The *BRCA1* gene encodes a 220 kDa nuclear phosphoprotein (3). A growing body of experimental evidence generally supports the notion that *BRCA1* is a caretaker gene whose encoded product functions in the maintenance of global genome stability (4-6). According to

this model, mutational inactivation of BRCA1 is accompanied not by direct promotion of tumor initiation but, rather, by widespread genetic instability and an increase in the mutation rates of all genes, including tumor suppressor genes and oncogenes. While the precise biochemical basis for its proposed caretaker function remains unknown, BRCA1 has nonetheless been implicated in both the regulation of transcription and the repair of damaged DNA.

With respect to transcription control, several lines of evidence support a direct role for BRCA1 in this process. First, the carboxyl-terminus of BRCA1 exhibits an inherent transactivation function sensitive to cancer-predisposing mutations (7-9). Second, BRCA1 has been identified as a component of the RNA polymerase II holoenzyme (10). Third, BRCA1 has been reported to interact with a variety of transcriptional activator and/or repressor proteins (11). Finally, BRCA1 has been reported to activate transcription of genes that encode activities involved in DNA damage-induced cell cycle arrest and/or apoptosis. These include the cyclin-dependent kinase inhibitor *p21* and the **G**rowth **A**rrest and **D**NA **D**amage-inducible **45** (***GADD45***) genes that function in G_1/S and/or G_2/M checkpoint control, and the *bax* gene that functions in DNA damage-induced apoptosis (12-15). Collectively, these observations imply a role for BRCA1 in mediation of DNA damage-induced cell cycle arrest and/or apoptosis through control of gene transcription.

A significant body of experimental evidence also implicates BRCA1 in DNA double-strand break repair. First, BRCA1 is known to undergo alterations in its phosphorylation status and subcellular localization in response to DNA damage (16). Second, *brca1*-deficient mouse embryonic stem cells are defective in the repair of both oxidative DNA damage by transcription-coupled processes and chromosomal double-strand breaks by homologous recombination (6,17). Third, *brca1*-mutant mouse embryo fibroblasts are characterized by genetic instability through

improper regulation of centrosome duplication and defective G_2/M checkpoint control (6). Finally, BRCA1 interacts physically and functionally with the Rad50/Mre11/NBS1 protein complex that participates in DNA damage detection, cell cycle checkpoint activation, and repair of DNA double-strand breaks (19).

Thus, while BRCA1 likely participates in the control of transcription and DNA double-strand break repair by virtue of its association with the RNA polymerase II holoenzyme and the Rad50/Mre11/NBS1 complex, respectively, the precise physical and functional relationship between these two sets of interacting proteins remains to be fully defined. For example, it is not presently clear whether these transcription and repair assemblies exist together in a single large complex or, alternatively, in distinct complexes with dedicated activities. With this issue in mind, we have undertaken the biochemical fractionation of human cell extracts to begin to decipher the protein networks through which BRCA1 functions. Our findings demonstrate that BRCA1, together with Rad50 and NBS1, can be isolated in distinct multiprotein complexes characterized by the stable association of these proteins with either transcription or repair activities. These results provide the first evidence for an association of Rad50 and NBS1 with the RNA polymerase II holoenzyme, and raise the possibility that these two proteins, along with BRCA1, represent a common core through which transcription and DNA repair activities may be linked within the cell.

EXPERIMENTAL PROCEDURES

Protein Purification - HeLa cell nuclear extract (~725 mg) was applied to Cibacron Hi-Trap Blue Sepharose (Amersham Pharmacia) at a concentration of 4.5 mg/ml protein (total of 7 X 5 ml columns; 103.5 mg protein/column) in 0.1M KCl D buffer (20). Columns were washed with

four column volumes of 0.1M KCl D buffer and bound proteins subsequently eluted with a linear gradient of 0.1-1.5M KCl in D buffer over a total volume of 40 ml. Blue Sepharose fractions containing the peaks of hSur2 (fractions 5-7) and Mrre11 (fractions 13-15) as determined by immunoblot analysis were pooled separately, dialyzed into 0.1M KCl D buffer, and processed in parallel as follows. Dialyzed Blue Sepharose fractions were applied to DEAE-Sepharose (10 mg protein/ml of resin) in 0.1M KCl D buffer. Columns were washed with four column volumes of 0.1M KCl D buffer and step-eluted with 0.3M KCl D buffer. Individual DEAE-Sepharose fractions containing the peaks of BRCA1, Rad50, and NBS1 along with either hSur2 or Mre11 proteins as determined by immunoblot analysis were pooled to a final concentration of 4 mg/ml and subjected to Superose 6 gel filtration chromatography (2 ml per 16 X 500 mm column). Individual Superose 6 column fractions were analyzed by immunoblot analysis and fractions corresponding to selected peaks as indicated were pooled, concentrated on phosphocellulose P-11 using a 0.6M KCl step elution, and subjected to immunoprecipitation analyses.

Antibody Immunoprecipitation - Monoclonal antibodies specific for the RNA polymerase II large subunit CTD (8WG16; ref. 21), human Rad50 (13B3; ref. 19), human p53 (PAb421; ref. 28), and Glutathione S-Transferase (8G11; ref. 19) were individually covalently coupled to protein G-Sepharose using dimethlylpimelimidate (29). Superose 6 column fractions containing peaks of BRCA1, Rad50, RNA polymerase II, and Mediator proteins were pooled and incubated in parallel with either 8WG16 (specific) or PAb421 (non-specific control) antibody columns in 0.3 M KCl (1/2) D buffer [(1/2) D buffer is 20 mM HEPES, pH 7.9; 0.2 mM EDTA; 10% glycerol; 5 mM β-mercaptoethanol] for 6 hours at 4° C. Superose 6 column fractions containing peaks of BRCA1, Rad50, NBS1, and Mre11 were pooled and incubated in parallel with either 13B3 (specific) or 8G11 (non-specific control) antibody columns in 0.3 M KCl (1/2) D buffer for 6

hours at hours 4° C. Column matrices were washed three times with ten column volumes of 0.3 M KCl (1/2) D buffer, once with ten column volumes of 0.1M KCl (1/2) D buffer, and eluted with one column volume of 0.1 M glycine (pH 2.0). Column eluates were neutralized, subjected to SDS-10%PAGE, and characterized by silver stain or immunoblot analysis as indicated.

RESULTS

Human HeLa cell nuclear extract was fractionated over Cibacron Hi-Trap Blue Sepharose using a linear gradient of KCl (0.1-1.5 M). Immunoblot analysis of the pre- and post-column extract revealed that the bulk of BRCA1, as well as Rad50, Mre11, NBS1, RNA polymerase II, and human Mediator proteins hSur2 and CDK8 (20) bound quantitatively to the Blue Sepharose matrix (Fig. 1; data not shown). Immunoblot analysis of individual chromatographic fractions revealed a broad elution profile for BRCA1, Rad50, and NBS1 (Fig. 1). By contrast, hSur2 eluted early in the gradient, peaking at ~0.45 M KCl, while Mre11 eluted later, peaking at ~1.2 M KCl.

The presence of BRCA1, Rad50, and NBS1 in distinct chromatographic fractions corresponding to the peaks of a Mediator subunit on one hand (hSur2) and a double-strand break repair protein on the other (Mre11) led us to ask whether the three former proteins could be isolated in stable association with either of the latter two proteins. To address this question, Blue Sepharose fractions corresponding to the peaks of hSur2 (Fig 1; fractions 5-7) and Mre11 (Fig. 1; fractions 13-15) were pooled separately and subjected in parallel to further fractionation first by DEAE-Sepharose anion exchange and subsequently by Superose 6 gel filtration chromatography.

Immunoblot analysis revealed co-elution of hSur2, BRCA1, Rad50, and NBS1 during DEAE-Sepharose chromatography of Blue-Sepharose fractions 5-7 (data not shown; Fig. 2a,

lane 1). Superose 6 chromatography of peak DEAE-Sepharose fractions revealed co-elution of BRCA1, Rad50, and NBS1 in one major peak within the included volume (Fig. 2a; fractions 48-51), and in a second peak which corresponds to the excluded (void) volume of the Superose 6 column (Fig. 2a; fractions 39-42). The included and excluded Superose 6 peaks of BRCA1, Rad50, and NBS1 could be distinguished by the absence or presence of additional proteins. Specifically, the included peak, which eluted well ahead of the 670 kDa thyroglobulin marker, is characterized by the additional presence of RNA polymerase II holoenzyme components, including the RNA polymerase II large subunit, RPB1, and human Mediator proteins CDK8, Cyclin C, and Med7. Significantly, no Mre11 protein could be detected in these fractions. The excluded peak, by contrast, was characterized by the presence, in addition to BRCA1, Rad50, and NBS1, of RNA polymerase II and a substoichiometric level of Mre11; however, little or no hSur2, CDK8, Cyclin C or Med7 could be detected. The presence of Mre11 within the excluded peak likely derives from trace amounts of a BRCA1, Rad50, NBS1, and Mre11-containing complex incompletely resolved in the initial Blue Sepharose fractionation step. The ability of Superose 6 to resolve this excluded peak containing Mre11 from an included peak of BRCA1, Rad50, and NBS1 in association with RNA polymerase II holoenzyme components raised the possibility that these two peaks represent stable and distinct multiprotein assemblies.

To determine if BRCA1, Rad50, and NBS1 all reside in a stable complex with RNA polymerase II holoenzyme components, individual Superose 6 column fractions corresponding to the included peaks of BRCA1, Rad50, NBS1, and holoenzyme components (Fig. 2a; fractions 48-51) were pooled and subjected to immunoprecipitation using an RNA polymerase II large subunit (RPB1)-specific monoclonal antibody, 8WG16 (21). Immunoblot analysis revealed specific co-immunoprecipitation of BRCA1, Rad50, and NBS1 along with RNA polymerase II

and Mediator proteins CDK8, Cyclin C, and Med7 (Fig. 2b). This result demonstrates that these proteins all reside in a single, large molecular-size complex, which likely corresponds to the RNA polymerase II holoenzyme.

To begin to characterize the protein complex that contains BRCA1 in association with Mre11, Blue Sepharose fractions 13-15 were pooled and applied to a DEAE-Sepharose anion exchange resin. Immunoblot analysis of DEAE fractions revealed co-elution of BRCA1, Rad50, and NBS1 along with Mre11 in a 0.3M KCl step elution (data not shown). Superose 6 chromatography of peak DEAE-Sepharose fractions revealed co-elution of BRCA1, Rad50, NBS1, and Mre11 in one peak within the included volume (Fig. 3a; fractions 47-50), and in a second peak which corresponds to the excluded (void) volume of the Superose 6 column (Fig. 3a; fractions 40-43). The included and excluded Superose 6 peaks of BRCA1, Rad50, NBS1, and Mre11 could be distinguished by the absence or presence of additional proteins. Specifically, the excluded peak is characterized by the additional presence of small amounts of ATM and Rad51, although the bulk of ATM eluted in fractions corresponding to a molecular size of ~ 600-700 kDa, while the bulk of Rad51 eluted in fractions corresponding to the expected size of its monomeric form. We consider it likely that the excluded peak represents an insoluble protein aggregate, since BRCA1, Rad50, and NBS1 exhibit a propensity to precipitate from solution (T.G. Boyer, unpublished data). Alternatively, the excluded peak could represent either an extremely large soluble protein complex or a protein/nucleic acid complex. Because these issues have not yet been thoroughly resolved, we have pursued analysis of Superose 6 fractions corresponding to the included peak of the BRCA1, Rad50, NBS1, and Mre11 proteins.

To determine if BRCA1, Rad50, NBS1, and Mre11 all reside in a stable complex, individual Superose 6 column fractions corresponding to the included peaks of the BRCA1,

Rad50, NBS1, and Mre11 proteins (Fig 3a; fractions 47-50) were subjected to immunoprecipitation using a Rad50-specific monoclonal antibody, 13B3. Immunoblot analysis revealed specific co-immunoprecipitation of BRCA1, Rad50, and NBS1 along with Mre11 and at least 10 additional polypeptides (Fig. 3b). This result demonstrates that these proteins all reside in a single, large molecular-size complex.

DISCUSSION

We have undertaken the biochemical fractionation of human cell extracts in an initial effort to decipher the protein networks involved in BRCA1 function. Previous studies have implicated this tumor suppressor in both the control of transcription and the repair of damaged DNA (11). Consistent with these proposed functional roles, biochemical and protein interaction analyses have demonstrated direct and specific interaction of BRCA1 with both transcription and DNA repair activities. For example, it has been demonstrated that BRCA1 interacts individually with the RNA polymerase II holoenzyme, with Rad51, and with the Rad50/Mre11/NBS1 DNA double-strand break repair complex (10,19,22). Thus, it appears likely that BRCA1 participates in a diverse range of DNA transactions by virtue of its association with these specific transcription and repair complexes. However, at present, the physical and functional relationship between these protein assemblies has remained undefined. For example, it is not presently clear whether these transcription and repair assemblies exist together in a single large complex or, alternatively, in distinct complexes of dedicated activity. We provide biochemical evidence to suggest that these activities, while perhaps linked within the cell, may nonetheless be isolated as distinct and stable macromolecular assemblies. The proteins common to both of these identified complexes are BRCA1, Rad50, and NBS1. We propose a model in which these proteins

function as an assembly interface for activities involved in transcription, repair, and possibly other cellular processes (Fig. 4).

The simultaneous presence of BRCA1, Rad50, and NBS1 in distinct multiprotein complexes with apparent transcription and repair functions provides a basis for the functional, and perhaps physical, linkage of these activities within the cell. We envision two alternative possibilities for the association of these two activities. First, these transcription and repair assemblies could represent components of a larger complex within the cell that has undergone fractionation in vitro. Alternatively, these complexes could represent distinct assemblies in vivo which, by virtue of shared subunits, are linked functionally. For example, a dynamic redistribution of BRCA1, Rad50, and NBS1 among transcription and repair complexes could effect global alterations in these activities sufficient to meet the immediate physiological demands of the cell. The biochemical basis for such redistribution could involve phosphorylation, a notion consistent with observed alterations in the phosphorylation status and subcellular localization of BRCA1 as a consequence of cell cycle progression or cellular DNA damage (3,16). Detailed biochemical characterization of BRCA1 in association with transcription and repair complexes should reveal whether post-translational modification represents a determinant of its interaction properties.

Our identification of both Rad50 and NBS1 in association with RNA polymerase II and transcriptional Mediator proteins represents, to our knowledge, the first demonstration of an interaction of these proteins with the RNA polymerase II holoenzyme. While this complex has not yet been tested functionally for holoenzyme activity, we note the quantitative association of Mediator proteins with the form of RNA polymerase II found in association with Rad50 and NBS1. As specific Mediator proteins have been demonstrated to be globally required for RNA

polymerase II-directed transcription (23,24), we consider it likely that the RNA polymerase II form found in association with BRCA1, Rad50, and NBS1 represents functional holoenzyme.

The presence of NBS1 in the RNA polymerase II holoenzyme suggests that this protein may, like BRCA1, be involved dually in transcription and DNA repair. The precise role of NBS1, if any, in the control of transcription remains to be defined. However, such a role would not be entirely inconsistent with the observed pleiotropic features associated with an absence of NBS1 activity in Nijmegen breakage syndrome, which include microcephaly, growth and mental retardation, chromosomal instability, immunodeficiency, and a high incidence of hematopoietic malignancy (25). While NBS1 has, apart from its direct role in repair, been implicated in checkpoint control through regulation of ionizing radiation-induced p53 protein levels (26,27), a more direct role for NBS1 in control of gene transcription cannot be ruled out at present. Future analyses should serve to clarify whether and how NBS1 functions in association with the RNA polymerase II machinery to effect alterations in gene-specific transcription.

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FIGURE LEGENDS

Figure 1. BRCA1 and Rad50 co-elute from Cibacron Blue Sepharose with both transcriptional Mediator and DNA repair proteins. HeLa nuclear extract was applied to a Hi-Trap Blue column at 0.1M KCl, and bound proteins were eluted with a linear gradient of 0.1-1.5M KCl. Aliquots of the on-put nuclear extract (NEXT), the column flow-through (BS FT), and individual chromatographic fractions (numbered) were analyzed by immunoblot analysis using antibodies specific for the proteins indicated on the left of the blot. Fractions corresponding to the peaks of hSur2 (fractions 5-7) and Mre11 (fractions 13-15) were pooled separately and processed in parallel for further chromatographic analyses as indicated.

Figure 2. BRCA1, Rad50, and NBS1 reside in stable association with RNA polymerase II holoenzyme components. a. Superose 6 gel filtration profile of Blue Sepharose and DEAE-Sepharose fractionated proteins. Pooled Blue Sepharose fractions 5-7 (from Fig.1) were applied to DEAE-Sepharose and peak fractions from a 0.3M KCl step elution containing BRCA1, Rad50, and NBS1 were pooled and subjected to gel filtration on Superose 6. Aliquots of the on-put DEAE fraction (Load) and individual column fractions (numbered) were analyzed by immunoblot analysis using antibodies specific for the proteins indicated on the left. Downward-pointing arrows indicate the positions of marker protein peaks. Fractions corresponding to the excluded (void) and included volume of the Superose 6 column are indicated. **b.** Co-immunoprecipitation of BRCA1 and NBS1 with RNA polymerase II holoenzyme components. Superose 6 fractions 48-51 (from **a**) were pooled, concentrated on phosphocellulose using a 0.6M KCl step elution (lanes 2 and 5) and subjected to immunoprecipitation with an RNA

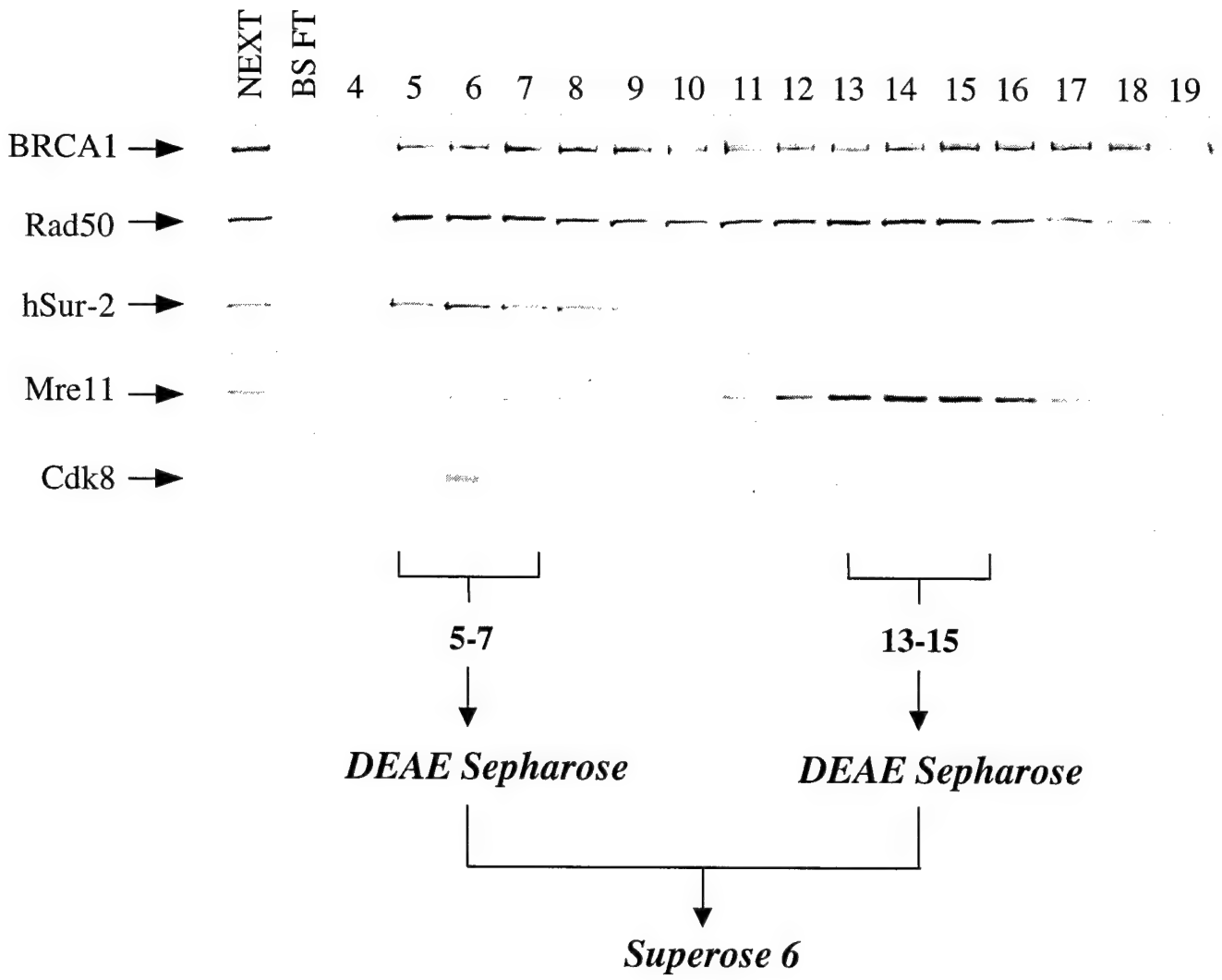
polymerase II large subunit (RPB1)-specific monoclonal antibody 8WG16 (lanes 4 and 7), or a p53-specific monoclonal antibody PAb421 (lanes 3 and 6) as a negative control. Immunoprecipitated proteins were eluted from covalently-coupled antibody columns with glycine, subjected to SDS-10%PAGE, and processed either by silver staining (lanes 1-4) or immunoblot analysis (lanes 5-7) with antibodies specific for the proteins indicated on the right. Molecular weight markers are indicated on the left. Lane 1 represents highly purified core RNA polymerase II, only the two largest subunits of which stained visibly on this gel.

Figure 3. BRCA1, Rad50, and NBS1 reside in stable association with Mre11 and additional polypeptides. a. Superose 6 gel filtration chromatography of Blue Sepharose and DEAE-Sepharose fractionated proteins. Pooled Blue Sepharose fractions 13-15 (from Fig. 1) were applied to DEAE-Sepharose and peak fractions from a 0.3M KCl step elution containing BRCA1, Rad50, NBS1, and Mre11 were pooled and subjected to gel filtration on Superose 6. Aliquots of individual column fractions (numbered) were analyzed by immunoblot analysis using antibodies specific for the proteins indicated on the left. Downward-pointing arrows indicate the positions of marker protein peaks. Fractions corresponding to the excluded (void) and included volume of the Superose 6 column are indicated. **b.** Co-immunoprecipitation of BRCA1, Rad50, and Mre11 along with additional polypeptides. Superose 6 fractions 47-50 (from **a**) were pooled, concentrated on phosphocellulose using a 0.5M KCl step elution (lanes 1 and 4), and subjected to immunoprecipitation with a Rad50-specific monoclonal antibody 13B3 (lanes 2 and 6), or a GST-specific monoclonal antibody 8G11 (lanes 3 and 5) as a negative control. Immunoprecipitated proteins were eluted from covalently-coupled antibody columns with glycine, subjected to SDS-10%PAGE, and processed either by silver staining (lanes 1-3) or

immunoblot analysis (lanes 4-6) with the antibodies specific for the proteins indicated on the right. Molecular weight markers are indicated on the left. The ~95 kDa protein specifically co-immunoprecipitated with anti-Rad50 antibody has been confirmed by immunoblot analysis to be NBS1, as indicated by the elongated arrow.

Figure 4. Schematic model for association of BRCA1, Rad50, and NBS1 with transcription and DNA repair complexes. In the absence of a specific DNA repair signal(s), BRCA1, Rad50, and NBS1 associate with RNA polymerase II holoenzyme components. As shown here, hypophosphorylated BRCA1 associates with the RNA polymerase II holoenzyme. At present, the specific cell signal(s) that directs BRCA1, Rad50, and NBS1 to the RNA polymerase II holoenzyme are unknown. Cell cycle- and/or DNA damage-induced signaling results in the phosphorylation of BRCA1 which, with Rad50 and NBS1, associates with Mre11 and the DNA double-strand break repair machinery.

Fig. 1


NEXT
BS FT
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BRCA1
Rad50
hSur-2
Mre11
Cdk8
5-7
13-15
DEAE Sepharose
DEAE Sepharose
Superose 6

Fig. 2

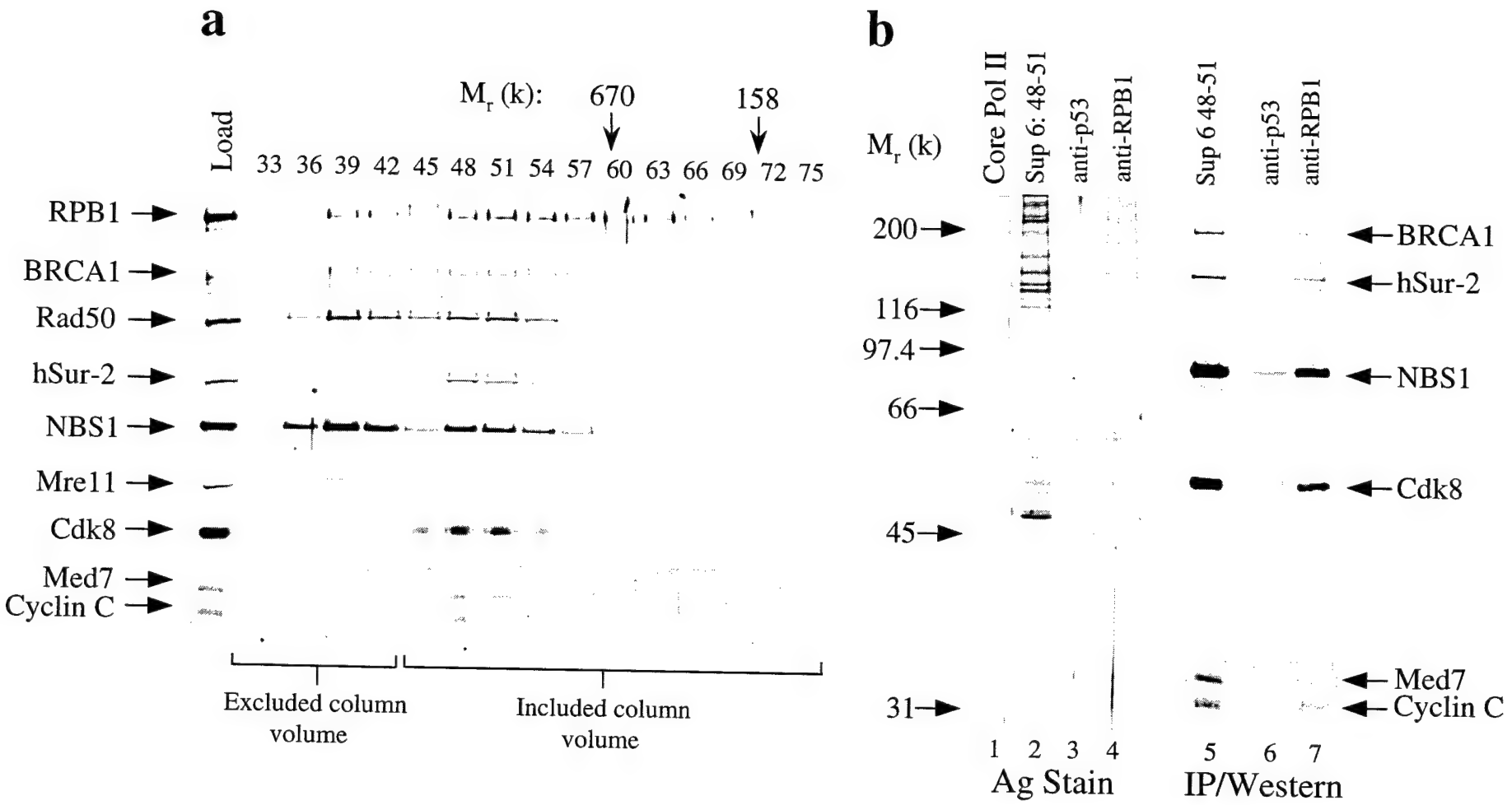

a
Load
M$_r$ (k):
670
158
33 36 39 42 45 48 51 54 57 60 63 66 69 72 75
RPB1
BRCA1
Rad50
hSur-2
NBS1
Mre11
Cdk8
Med7
Cyclin C
Excluded column volume
Included column volume
b
M$_r$ (k)
Core Pol II
Sup 6: 48-51
anti-p53
anti-RPB1
Sup 6 48-51
anti-p53
anti-RPB1
200
116
97.4
66
45
31
BRCA1
hSur-2
NBS1
Cdk8
Med7
Cyclin C
1 2 3 4
Ag Stain
5 6 7
IP/Western

Fig. 3

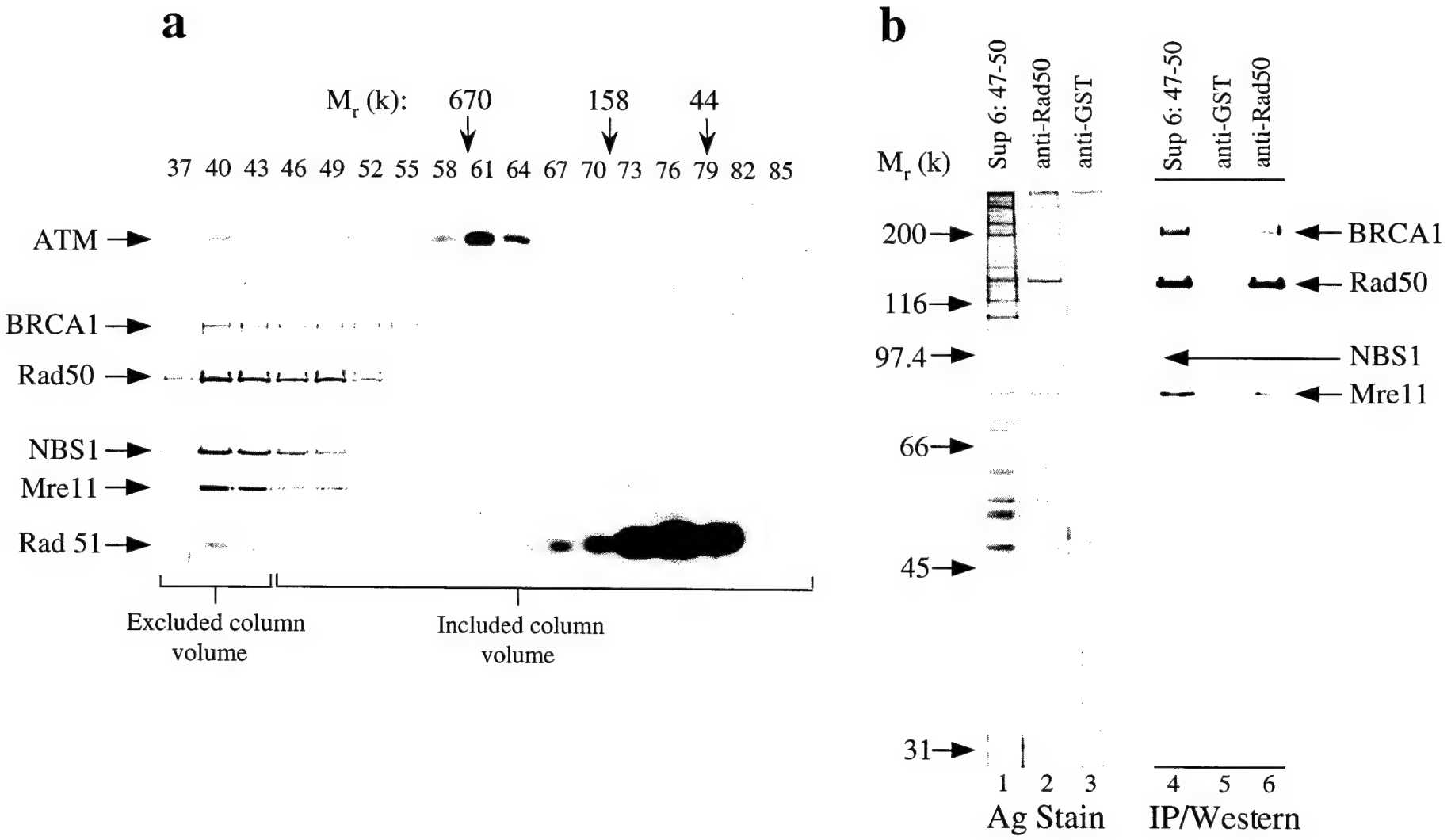
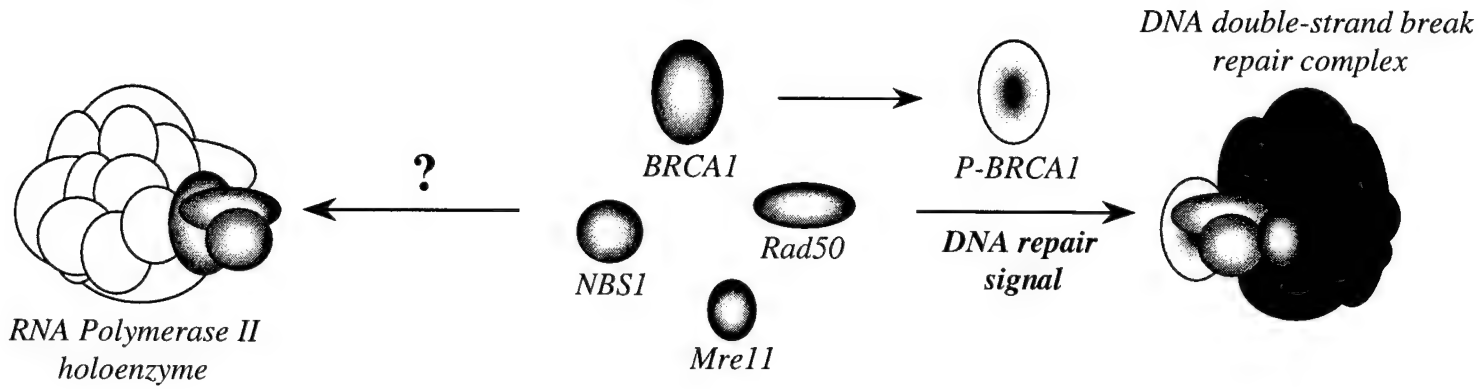

a
M$_r$ (k): 670 158 44
37 40 43 46 49 52 55 58 61 64 67 70 73 76 79 82 85
ATM
BRCA1
Rad50
NBS1
Mre11
Rad 51
Excluded column volume
Included column volume
b
M$_r$ (k)
Sup 6: 47-50
anti-Rad50
anti-GST
Sup 6: 47-50
anti-GST
anti-Rad50
200
116
97.4
66
45
31
BRCA1
Rad50
NBS1
Mre11
1 2 3
Ag Stain
4 5 6
IP/Western

Fig. 4


DNA double-strand break
repair complex
BRCA1
P-BRCA1
?
Rad50
NBS1
DNA repair
signal
Mre11
RNA Polymerase II
holoenzyme

BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor

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Mutational inactivation of BRCA1 confers a cumulative lifetime risk of breast and ovarian cancers. However, the underlying basis for the tissue-restricted tumor-suppressive properties of BRCA1 remains poorly defined. Here we show that BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor α (ERα), a principal determinant of the growth, differentiation, and normal functional status of breasts and ovaries. In Brca1-null mouse embryo fibroblasts and BRCA1-deficient human ovarian cancer cells, ERα exhibited ligand-independent transcriptional activity that was not observed in Brca1-proficient cells. Ectopic expression in Brca1-deficient cells of wild-type BRCA1, but not clinically validated BRCA1 missense mutants, restored ligand-independent repression of ERα in a manner dependent upon apparent histone deacetylase activity. In estrogen-dependent human breast cancer cells, chromatin immunoprecipitation analysis revealed the association of BRCA1 with ERα at endogenous estrogen-response elements before, but not after estrogen stimulation. Collectively, these results reveal BRCA1 to be a ligand-reversible barrier to transcriptional activation by unliganded promoter-bound ERα and suggest a possible mechanism by which functional inactivation of BRCA1 could promote tumorigenesis through inappropriate hormonal regulation of mammary and ovarian epithelial cell proliferation.

Germline inactivation of the gene that encodes BRCA1 represents a predisposing genetic factor in ≈15–45% of hereditary breast cancers, and minimally 80% of combined hereditary breast and ovarian cancer cases (1). Functionally, BRCA1 has been implicated in the maintenance of global genome stability (2–4), and the underlying basis for this activity likely derives from its central role in the cellular response to DNA damage, wherein it controls both DNA damage repair and the transcription of DNA damage-inducible genes (5–14).

Because the DNA damage-induced signaling pathways that converge on BRCA1 are likely to be conserved in most cell types, BRCA1 is likely to occupy a fundamental and universally conserved role in the mammalian DNA damage response. Nonetheless, germ-line inactivation of BRCA1 leads predominantly to cancer of the breast and ovary, and the underlying basis for its tissue-restricted tumor-suppressive properties thus remains undefined.

At least two hypotheses have been proposed to explain the tissue-specific nature of BRCA1-mediated tumor suppression, both of which invoke a role for estrogen in either the initiation or promotion of tumor formation (15). According to one model, the tissue-specific tumor-suppressive properties of BRCA1 derive, at least in part, from its response to tissue-specific DNA damage. In this regard, certain oxidative metabolites of estrogen itself have been documented to be genotoxic in nature (16), and BRCA1 may therefore play a role in protecting breast and ovarian tissue from estrogen-induced DNA damage.

A second model, not mutually exclusive with the one described above, to account for the this tissue-specific tumor-suppressive function invokes a role for BRCA1 in the modulation of estrogen signaling pathways and, hence, the expression of hormone-responsive genes. In this regard, BRCA1 has been reported to inhibit estrogen-dependent transactivation by the estrogen receptor α (ERα) through its direct interaction with ERα (17, 18). BRCA1 has also been reported to enhance androgen-dependent transactivation by the androgen receptor, allelic variants of which modify cancer penetrance in BRCA1 mutation carriers (19–21). Based on its postulated role in the control of nuclear hormone signaling pathways, BRCA1 could therefore influence epithelial cell proliferation and, by implication, cancer risk in tissues such as breast and ovary.

Herein, we describe a role for BRCA1 in mediating ligand-independent transcriptional repression of the ERα. Initial efforts to elucidate the mechanistic basis for this repression reveal that BRCA1 represents a ligand-reversible barrier to transcriptional activation by unliganded promoter-bound ERα. These findings suggest a potential role for BRCA1 in the proliferative control of normal estrogen-regulated tissues and a potential basis by which its mutational inactivation could promote tumorigenesis through inappropriate hormonal responses.

BIOCHEMISTRY

Materials and Methods

Cell Culture. *p53−/−* (Brca1+/+) and *p53−/−; Brca1−/−* (Brca1−/−) mouse embryonic fibroblasts (MEFs) were cultured as described (14). Human MCF7 cells were maintained in DMEM supplemented with 10% FCS. Human BG-1-derived NEO1 and AS4 cell lines were maintained as described (22). Depletion of hormone ligands for nuclear/steroid receptor activation studies was achieved by cell culture in medium containing either 10% charcoal/dextran-treated serum (HyClone) or defined serum replacement 2 (Sigma).

Plasmids and Transfections. Transfection assays were performed by using the following conditions.

Reporter plasmids. Used at 0.5 μg each, including pTRE(F2)-TK-Luc, pGRE-TK-CAT, pERE-TK-Luc, or pPRE-TK-CAT (23); 0.5 μg of pGAL4-SV40-Luc containing five GAL4 DNA-binding sites upstream of the minimal simian virus 40 (SV40) promoter, driving expression of the luciferase reporter gene in the pGL2 vector (Promega); and 0.5 μg of pGAL4-E1B-Luc (24).

Receptor expression plasmids. Used at 1.0 μg each, including RSV-hTRβ, RSV-hGR, RSV-hERα, and RSV-hPRβ (23).

BRCA1 expression plasmids. Used at 1.0 μg each, including pcDNA3.1-BRCA1, pcDNA3.1-BRCA1-A1708E, pcDNA3.1-BRCA1-Q356R, and pcDNA3.1-BRCA1-A1708E/Q356R expressing either human wild-type BRCA1 or familial breast cancer-derived BRCA1 mutants (14).

Abbreviations: ERα, estrogen receptor α; MEF, mouse embryonic fibroblast; E2, 17β-estradiol; RT-PCR, reverse transcription–PCR; HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation; AF-1, N-terminal ligand-independent activation function; AF-2, C-terminal ligand-inducible activation function.

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Chimeric activators. Used at 1.0 μg of GAL4-ERα, generated by an amino-terminal fusion of ERα with the GAL4 DNA-binding domain in pM3 (25); 0.1 μg of pVP16-GAL4 or pVP16-GAL4-ERα containing ERα amino acids 251–595, as described (26).

MEFs (6×10^4) or BG-1 cells (2×10^5) cultured in ligand-free medium were transfected by Lipofectin-based methods under serum-free conditions. Culture medium was replaced with fresh ligand-free medium 24 h after transfection, and 10^{-7} M 17β-estradiol (E2) or 330 nM trichostatin A was added as indicated. Cells were harvested 48 h after transfection for luciferase assay as described (14) or chloramphenicol acetyltransferase (CAT) assay by liquid scintillation counting (Promega).

Reverse Transcription (RT)-PCR Analysis. BG-1-derived cells were cultured in ligand-free medium for at least 5 days, and treated with 10^{-7} M E2 for 1 h as indicated. Approximately 15 μg of total cellular RNA was subjected to semiquantitative RT-PCR analysis following a procedure previously described for estrogen-responsive genes (27, 28).

Chromatin Immunoprecipitation (ChIP). MCF7 cells were cultured in ligand-free medium for at least 5 days and treated with 10^{-7} E2 for 1 h as indicated. ChIP assays were performed as described (29).

Antibodies. Antibodies used for soluble and chromatin immunoprecipitations and immunoblot analyses were as follows: BRCA1 (mAb 6B4); ERα (rabbit polyclonal antibody HC-20 or mouse mAb D-12, Santa Cruz Biotechnology); CtIP (mAb 19E8); TFIIH p89 (rabbit polyclonal antibody S-19, Santa Cruz Biotechnology); glutathione *S*-transferase (MAb 8G11); RNA polymerase II large subunit (mAb 8WG16); cathepsin D (rabbit polyclonal antibody 06-467, Upstate Biotechnology, Lake Placid, NY); pS2 (mouse mAb V3030, Biomeda, Hayward, CA); human progesterone receptor β (mouse mAb PriB-30, Santa Cruz Biotechnology); p84 (mAb 5E10).

Results

BRCA1 has been shown to modulate the ligand-dependent transcriptional activity of specific members of the nuclear hormone receptor family (17–20). However, endogenous BRCA1 present in the transfected cell lines used in previous studies precluded analysis of the effect of BRCA1 on the ligand-independent function of these receptors. Therefore, to more directly assess the role of BRCA1 in nuclear receptor transactivation without competition from endogenous BRCA1, we analyzed a panel of nuclear receptors for their respective ligand-independent transcriptional activities in Brca1-nullizygous MEFs.

A set of minimal thymidine kinase (TK) promoters, each under control of distinct hormone-response elements specific for either the human thyroid receptor β (TRβ), the glucocorticoid receptor (GR), the ERα, or the progesterone receptor β (PRβ) were individually tested for their respective abilities to direct expression of a reporter gene in the absence or presence of each corresponding receptor (absent ligand) after transfection into Brca1-proficient (Brca1+/+) or Brca1-deficient (Brca1−/−) MEFs (14). Unexpectedly, we observed significant ligand-independent activation of reporter gene expression directed by both the progesterone receptor β and the ERα in Brca1-deficient MEFs compared with Brca1-proficient MEFs (Fig. 1*A*). By contrast, no ligand-independent stimulation of reporter activity directed by either the thyroid receptor β or the glucocorticoid receptor could be observed in Brca1-deficient MEFs (Fig. 1*A*). Interestingly, although E2 activated the ERα in both Brca1-proficient and Brca1-deficient MEFs, the relative level of induction observed in Brca1-deficient MEFs was diminished 2-fold

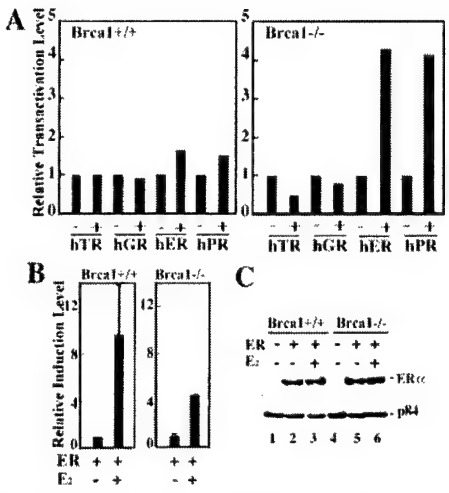

Fig. 1. BRCA1 mediates ligand-independent repression of the receptors for estrogen and progesterone. (*A*) Brca1+/+ and Brca1−/− MEFs in hormone-free media were transfected with reporter plasmids (pTK-Luc or pTK-CAT) carrying response elements specific for individual hormone receptors without (−) or with (+) plasmids expressing the human thyroid receptor β (hTR), glucocorticoid receptor (hGR), estrogen receptor α (hER), or progesterone receptor β (hPR). Transfections performed without (−) receptor expression plasmids were performed instead with a molar equivalent of the backbone expression plasmid pRSV. The relative transactivation level represents the fold-increase in transfected reporter gene activity measured in cells cotransfected with a specific receptor expression plasmid relative to the level of transfected reporter gene activity measured in cells cotransfected with the backbone pRSV expression plasmid. Reporter gene activity was first normalized to β-galactosidase activity obtained by cotransfection of an internal control pSV40-β-gal expression plasmid as described (14). Expression of the pSV40-β-gal plasmid was not affected by the absence of presence of BRCA1 or any of the nuclear hormone receptors analyzed (data not shown). (*B*) Brca1+/+ and Brca1−/− MEFs in estrogen-free media were transfected with pERE-TK-Luc carrying three copies of the consensus estrogen response element (ERE) with (+) pRSV-ERα in the absence (−) or presence (+) of E2 (10^{-7} M) before assay for luciferase activity. The relative induction level represents the relative transactivation level measured in the presence of E2 divided by the relative transactivation level measured in the absence of E2. (*C*) Brca1+/+ (lanes 1–3) and Brca1−/− (lanes 4–6) MEFs either untransfected (lanes 1 and 4) or transfected (lanes 2, 3, 5, and 6) with an ERα-expressing vector were lysed, and immunoprecipitated ERα was immunoblotted with ERα-specific antibodies (*Upper*). Immunoblot analysis of the nuclear matrix protein p84 (*Lower*) indicates that nearly equivalent amounts of each cell lysate were used in the immunoprecipitations.

relative to Brca1-proficient MEFs (Fig. 1*B*). We confirmed by immunoblot analysis that the transfected ERα was expressed equivalently in BRCA1-proficient and BRCA1-deficient MEFs, thus excluding the possibility that differences in receptor activity derive from differences in receptor protein expression (Fig. 1*C*).

Ectopic expression of wild-type BRCA1 in Brca1-deficient MEFs repressed ligand-independent activation directed by ERα (Fig. 2*A*). Likewise, a BRCA1 derivative carrying a familial breast cancer-derived missense mutation in the ring finger (C64G) also repressed ligand-independent activation by ERα (Fig. 2*A*). By contrast, BRCA1 derivatives carrying familial breast cancer-derived missense mutations in either an exon 11-encoded region that binds Rad50 and the transcriptional repressor ZBRK1 (Q356R) or the C-terminal BRCT domain (A1708E) abolished the ability of BRCA1 to repress ligand-independent transactivation directed by ERα (Fig. 2*A*). Differences in the transcriptional repression activities of the various BRCA1 mutant derivatives could not be attributed to differences in their respective levels of expression because each of the BRCA1 mutant derivatives was expressed at a level comparable

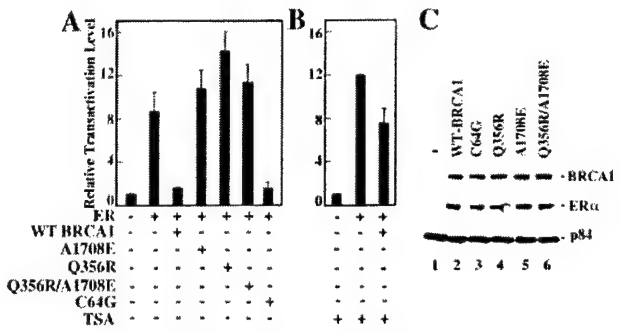

Fig. 2. Ectopic expression of wild-type BRCA1 in Brca1-deficient MEFs restores ligand-independent repression of ERα transactivation in a histone deacetylase (HDAC)-dependent manner. (*A* and *B*) Brca1−/− MEFs in estrogen-free media were transfected with pERE-TK-Luc without (−) or with (+) pRSV-ERα, pCDNA3.1-BRCA1 expressing wild-type human BRCA1 (WT), or pCDNA3.1-BRCA1 derivatives bearing missense mutants A1708E, Q356R, A1708E/Q356R, or C64G before assay for luciferase activity. Where indicated, trichostatin A (TSA; 330 nM) was also included. (*C*) Brca1−/− MEFs in estrogen-free media were untransfected (lane 1) or cotransfected with expression vectors for ERα and either wild-type BRCA1 (lane 2) or various BRCA1 mutant derivatives (lanes 3–6) as indicated. Cells were lysed, and immunoprecipitated BRCA1 and ERα were subjected to immunoblot analysis using antibodies specific for BRCA1 (*Top*) or ERα (*Middle*). Immunoblot analysis of the nuclear matrix protein p84 (*Bottom*) indicates that nearly equivalent amounts of each cell lysate were used in the immunoprecipitations.

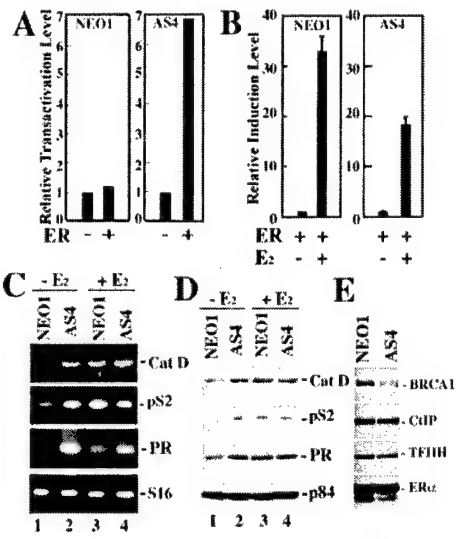

Fig. 3. Reduced BRCA1 expression in BG-1 human ovarian adenocarcinoma cells is accompanied by increases in estrogen-independent expression of estrogen-responsive genes. (*A*) Retroviral vector-infected (NEO1) and BRCA1 antisense-infected (AS4) BG-1 cell clones in estrogen-free media were transfected with pERE-TK-Luc without (−) or with (+) pRSV-ERα before assay for luciferase activity. (*B*) NEO1 and AS4 cells in estrogen-free media were transfected with pERE-TK-Luc with (+) pRSV-ERα in the absence (−) or presence (+) of E2 (10^{-7} M) before assay for luciferase activity. (*C*) NEO1 (lanes 1 and 3) or AS4 (lanes 2 and 4) cells in estrogen-free media were either untreated (lanes 1 and 2) or treated (lanes 3 and 4) with E2 (10^{-7} M) for 1 h. Cells were harvested and processed for semiquantitative RT-PCR analysis using primers specific for the estrogen-responsive cathepsin D (Cat D), pS2, and progesterone receptor genes, as well as the estrogen-nonresponsive ribosomal S16 gene. (*D*) NEO1 (lanes 1 and 3) or AS4 (lanes 2 and 4) cells (5×10^6) in estrogen-free media were either untreated (lanes 1 and 2) or treated (lanes 3 and 4) with E2 (10^{-7} M) for 24 h. Culture medium was concentrated 10-fold by using a Centriprep YM-3 device, and 1/10th of the concentrate was resolved by SDS/15%PAGE and processed for immunoblot analysis using antibodies specific for pS2. Cells were also lysed in RIPA buffer, and 1/10th of the lysate was subjected to immunoblot analysis using antibodies specific for progesterone receptor β (PR), cathepsin D (Cat D), or nuclear matrix protein p84, which served as an internal loading control. (*E*) Whole cell lysates derived from NEO1 and AS4 cells were resolved by SDS/10%PAGE and processed for immunoblot analysis using antibodies specific for BRCA1, CtIP, and the p89 subunit of the transcription factor IIH (TFIIH), the latter two of which served as internal loading controls. The ERα-positive status of these cells was verified by using an ERα-specific rabbit polyclonal antibody. Densitometric quantitation of the immunoblot and normalization to the CtIP and TFIIH signals revealed BRCA1 expression to be reduced by 70% in AS4 cells compared with NEO1 cells.

to wild-type BRCA1 (Fig. 2*C*). BRCA1-mediated, ligand-independent repression of ERα was largely reversed by trichostatin A, implicating histone deacetylase (HDAC) activity in this process (Fig. 2*B*). Collectively, these results reveal a function for BRCA1 as a repressor of ligand-independent, ERα-mediated transactivation.

To confirm these results in a biologically relevant cell type, we analyzed the ligand-independent activity of ERα in human ovarian adenocarcinoma BG-1 cells, which are ERα-positive and estrogen-dependent for growth (30). Previously, Annab *et al.* (22) described the generation of independent BG-1 clonal cell lines that support stably reduced BRCA1 mRNA and protein levels by retroviral-mediated BRCA1 antisense delivery. We tested the ability of ERα to direct ligand-independent transcription of the ERE-TK-Luc reporter gene after transfection into either a control retroviral vector-infected BG-1 clonal cell line (NEO1) or, alternatively, a BRCA1 antisense-infected BG-1 clonal cell line (AS4) exhibiting severely reduced BRCA1 expression levels (Fig. 3*E*; ref. 22). Consistent with the results obtained in MEF cells, ERα exhibited significantly increased ligand-independent activity in BRCA1-deficient AS4 cells compared with BRCA1-proficient NEO1 cells (Fig. 3*A*). We also observed a 2-fold reduction in the relative level of E2-mediated induction of reporter gene activity in AS4 cells compared with NEO1 cells, once again consistent with the results obtained in MEF cells (Fig. 3*B*). These results confirm that in a biologically relevant epithelial cell type, BRCA1 can mediate repression of ligand-independent ERα transactivation activity.

To determine whether the reduced BRCA1 expression levels in AS4 cells could be correlated with an increase in the ligand-independent expression of endogenous estrogen-responsive genes, we performed a direct comparative analysis of NEO1 and AS4 cells with respect to their ligand-independent expression of several estrogen-responsive genes. Individual monolayer cultures of NEO1 and AS4 cells were grown in the absence of estrogen for 5 days followed by the addition of either no hormone or, alternatively, E2 (10^{-7} M) for 1 h. Subsequently, cells were harvested and analyzed by semiquantitative RT-PCR for the expression levels of the endogenous estrogen-responsive pS2, cathepsin D, and progesterone receptor genes.

Relative to the expression level of an internal control ribosomal S16 gene, we observed increases in the ligand-independent expression levels of the pS2, cathepsin D, and progesterone receptor genes of 3-, 5-, and 9-fold, respectively, in BRCA1-deficient AS4 cells compared with BRCA1-proficient NEO1 cells (Fig. 3*C*). Interestingly, although the addition of E2 stimulated transcription of the pS2, cathepsin D, and the progesterone receptor genes in NEO1 cells, no such E2-dependent increase in the transcription of these genes could be observed in AS4 cells (Fig. 3*C*). Qualitatively similar results were observed at the protein level by immunoblot analysis. Relative to the level of an internal control protein (nuclear matrix protein p84), E2-independent increases in the steady-state levels of the pS2, cathepsin D, and progesterone receptor proteins could be observed in AS4 cells compared with NEO1 cells (Fig. 3*D*). Furthermore, although the addition of E2 elevated the steady-

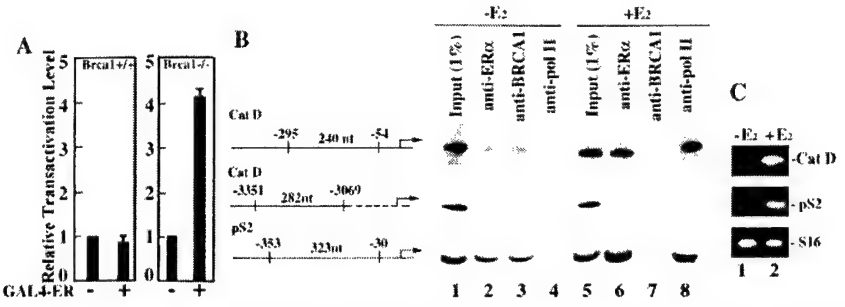

Fig. 4. BRCA1 represses unliganded promoter-bound ERα-mediated transactivation. (*A*) Brca1+/+ and Brca1−/− MEFs were transfected with a pGAL4-SV40-Luc reporter plasmid either without (−) or with (+) a pGAL4-ERα expression plasmid before assay for luciferase activity. (*B*) Schematic diagram of the cathepsin D (Cat D) and pS2 gene regions targeted for ChIP analysis. Negative numbers refer to sequence coordinates that delimit PCR amplicons defined by gene-specific primer pairs relative to the transcription initiation site (right-angled arrow). Numbered nucleotides (nt) refer to the expected sizes of PCR-amplified products. MCF-7 cells, cultured the absence of estrogen, were treated without (−E2) or with (+E2) E2 (10^{-7} M) for 1 h. Soluble chromatin was prepared and subjected to immunoprecipitation by using monoclonal antibodies specific for ERα (anti-ERα), BRCA1 (anti-BRCA1), or the RNA polymerase II large subunit (anti-pol II). Immunoprecipitated DNA was PCR-amplified by using primers that span the indicated regions of the cathepsin D and pS2 gene promoters. Input (1%) of the soluble chromatin subjected to immunoprecipitation was PCR-amplified directly by using each primer pair as indicated. (*C*) MCF-7 cells, cultured in the absence of estrogen, were treated without (−E2) or with (+E2) E2 (10^{-7} M) for 1 h before harvest and processing for semiquantitative RT-PCR analysis using primers specific for the estrogen-responsive cathepsin D (Cat D) and pS2 genes, as well as the estrogen-nonresponsive ribosomal S16 gene.

state level of each of these proteins in NEO1 cells, no such E2-dependent increase could be observed in AS4 cells (Fig. 3*D*). Quantitative differences between RT-PCR and immunoblot analyses could reflect the influence of posttranscriptional regulatory processes. Nonetheless, RT-PCR and immunoblot analyses both reveal that the ligand-independent expression of endogenous ERα-target genes is increased in BRCA1-deficient cells. Collectively, these results implicate BRCA1 in the ligand-independent repression of endogenous estrogen-responsive genes.

To explore the mechanism by which BRCA1 mediates ligand-independent repression of ERα, we first determined whether BRCA1 could interact with unliganded ERα *in vivo* by coimmunoprecipitation of the two proteins in human breast cancer MCF7 cells cultured in the absence of estrogen. Consistent with previous results (18), BRCA1 could be specifically coimmunoprecipitated with unliganded ERα, thus demonstrating that the two proteins can interact *in vivo* in a ligand-independent manner (data not shown).

To explore the possibility that BRCA1 represses the transactivation function of promoter-bound, unliganded ERα, we first tested the effect of BRCA1 on the ligand-independent transcriptional activity of ERα tethered to the yeast GAL4 DNA-binding domain by using a reporter template bearing GAL4 DNA-binding sites. This approach permitted us to assess the effect of BRCA1 on the transactivation function of unliganded ERα independent of any effects that BRCA1 might have on the DNA-binding activity of unliganded ERα. GAL4-ERα was cotransfected along with a GAL4-SV40-luciferase reporter template into Brca1-proficient and Brca1-deficient MEFs. We observed significant ligand-independent stimulation of reporter activity in Brca1-deficient, but not in Brca1-proficient, MEFs (Fig. 4*A*), suggesting one mechanism by which BRCA1 mediates ligand-independent repression of ERα is through direct repression of the DNA-bound receptor.

To confirm this observation under biologically relevant conditions *in vivo*, we used ChIP analyses to determine whether BRCA1 can be recruited directly to estrogen-responsive promoters in the absence of ligand. MCF-7 cells were grown in the absence of estrogen for 5 days followed by the addition of either no hormone or, alternatively, E2 (10^{-7} M) for 1 h. Promoter occupancy before and after E2 treatment at the estrogen response elements within the endogenous pS2 and cathepsin D gene promoters by ERα, BRCA1, and RNA polymerase II was then monitored by ChIP using antibodies specific for each of the three proteins and semiquantitative PCR with primers flanking the estrogen response elements of the pS2 and cathepsin D promoters. In the absence of E2, ERα could be detected in association with both the pS2 and cathepsin D promoters, and this level was increased dramatically by the addition of E2 (Fig. 4*B*, lanes 2 and 6). Strikingly, we also observed pS2 and cathepsin D promoter occupancy by BRCA1 in the absence of E2, and a reduction in such occupancy after E2 treatment (Fig. 4*B*, lanes 3 and 7). By contrast, RNA polymerase II could be detected only following, but not before, E2 treatment, consistent with its ligand-dependent recruitment concomitant with transcriptional activation of the pS2 and cathepsin D genes (Fig. 4*B*, lanes 4 and 8 and *C*, lanes 1 and 2). The specificity of factor association within the estrogen-responsive region of the pS2 and cathepsin D promoters was confirmed by ChIP analysis using antibodies specific for ZBRK1, a sequence-specific DNA-binding transcriptional repressor that does not bind to pS2 or cathepsin D promoter sequences (14). ZBRK1-specific antibodies failed to immunoprecipitate pS2 and cathepsin D promoter sequences (data not shown). Further specificity of the ChIP assay was demonstrated by the inability to detect occupancy by ERα, BRCA1, or RNA polymerase II of a region ≈3 kb upstream of the cathepsin D promoter (Fig. 4*B*). These results thus reveal the association of BRCA1 with unliganded ERα at endogenous estrogen-responsive promoters under physiologically relevant conditions *in vivo*.

Like other steroid receptors, ERα contains two transactivation domains, an N-terminal ligand-independent activation function (AF-1) that is targeted by a variety of steroid-independent cell-signaling pathways, and a C-terminal ligand-inducible activation function (AF-2) that resides within the receptor ligand-binding domain (31, 32). Previous analyses of ERα suggest a model whereby repressive factors binding to sequences within its C-terminal ligand-binding domain repress constitutively active AF-1 in the absence of an agonist or in the presence of an antagonist (26, 33). To determine whether ligand-independent repression of ERα by BRCA1 is mediated through the ERα ligand-binding domain, we tested the ligand-independent activity of a VP16-GAL4-ERα receptor chimera after its expression in both BRCA1-proficient and BRCA1-deficient BG-1 clonal cell lines. This chimera encodes ERα amino acids 251–595, including the hinge region and the ligand-binding domain, fused C-terminally to the hybrid transactivator VP16-GAL4 (26).

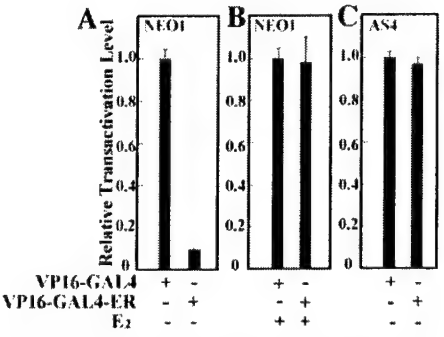

Fig. 5. VP16-GAL4-ERα exhibits hormone-dependent activity in BRCA1-proficient cells and constitutive activity in BRCA1-deficient cells. NEO1 (*A* and *B*) and AS4 (*C*) cells in estrogen-free media were transfected with a GAL4-E1B-Luc reporter plasmid along with (+) plasmids expressing either VP16-GAL4 or VP16-GAL4-ERα. Subsequently, transfected cells were either untreated (−) or treated (+) with E2 (10^{-7} M) before assay for luciferase activity.

Previously, deletion analysis of this receptor chimera revealed that constitutive VP16-GAL4-ERα activity could be recovered by the removal of sequences within the ligand-binding domain of the ERα moiety, thereby implicating the ERα ligand-binding domain in ligand-independent transcriptional repression of a neighboring constitutive activation domain (26). To determine whether this ligand-independent repression is mediated by BRCA1, we transfected the VP16-GAL4-ERα chimera along with a reporter template bearing GAL4 DNA binding sites into both BRCA1-proficient NEO1 cells and BRCA1-deficient AS4 cells. In NEO1 cells, the VP16-GAL4-ERα chimera exhibited minimal constitutive transactivation activity in the absence of E2; in response to E2, this level was dramatically increased to one approaching that of the potent VP16-GAL4 activator alone (Fig. 5 *A* and *B*). By contrast, in AS4 cells the VP16-GAL4-ERα chimera exhibited constitutive transactivation activity comparable to that exhibited by the VP16-GAL4 activator alone (Fig. 5*C*). The addition of E2 had a minimal effect on the elevated constitutive transactivation activity of the ERα chimera in AS4 cells (data not shown), suggesting that the principle effect of E2 is to override a ligand-independent barrier to the transactivation activity of the chimeric receptor. This barrier is present in NEO1 cells, but deficient in AS4 cells. Similar results were also observed by using isogenic Brca1-proficient and Brca1-deficient MEFs, eliminating the possibility that cell type-specific peculiarities contribute to the differential transactivation properties of the VP16-GAL4-ERα chimera in the presence and absence of BRCA1 (data not shown). Collectively, these results reveal the ERα ligand-binding domain to be a platform for the recruitment of BRCA1 from which the latter may confer ligand-independent repression on a linked activation domain. Hence, we conclude that BRCA1-mediated ligand-independent repression of ERα is likely to be mediated through the ERα ligand-binding domain.

Discussion

Recently, BRCA1 has been proposed to inhibit the ligand-dependent transcriptional activity of ERα through a direct interaction between the two proteins (18). Our current analysis of ERα transcriptional activity in Brca1-nullizygous MEFs revealed BRCA1 to be a ligand-reversible barrier to transcriptional activation by unliganded ERα. The biological relevance of this finding is further strengthened by the observation that BRCA1 also mediates ligand-independent repression of the ERα in human ovarian adenocarcinoma cells.

The underlying mechanism by which BRCA1 mediates ligand-independent repression of ERα transcriptional activity appears to involve targeted recruitment by unliganded, promoter-bound ERα of a BRCA1-associated HDAC activity. This conclusion is based first on the observation that the HDAC inhibitor trichostatin A can effectively reverse ligand-independent repression mediated by BRCA1 and, second, on the results of ChIP analyses, which revealed the association of unliganded ERα with BRCA1 on endogenous estrogen-response elements *in vivo*. A likely target of BRCA1-mediated ligand-independent ERα repression is the constitutive AF-1 activation domain within ERα. Previous studies have indicated that antagonist-bound AF-2 can repress AF-1 activity through the recruitment of the nuclear corepressor N-CoR (33), whereas the ligand-binding domain of unliganded ERα can repress a linked heterologous activation domain in a ligand-reversible manner, presumably by the recruitment of a soluble corepressor (26). Our observation that an estrogen-dependent VP16-GAL4 chimeric transactivator carrying the ERα ligand-binding domain exhibits constitutive activity in BRCA1-deficient, but not in BRCA1-proficient BG-1 cells, reveals the ERα ligand-binding domain to be a potential site of BRCA1 recruitment for ligand-independent repression of a linked activation domain. Hence, BRCA1 could be recruited to the ERα ligand-binding domain as part of a larger repression complex to silence AF-1 function in the absence of ligand. The recent report of a direct interaction between BRCA1 and the ERα ligand-binding domain (18) lends additional support to this model.

Should BRCA1 function to inhibit the ligand-dependent transcriptional activity of ERα (17, 18), it seems unlikely to do so through a mechanism that involves promoter-bound ERα. Our ChIP analysis revealed the association of BRCA1 with ERα at endogenous estrogen-response elements before, but not after, estrogen stimulation. Thus, we favor a model in which BRCA1, along with an associated corepressor(s) that minimally includes an HDAC activity, is recruited by unliganded, promoter-bound ERα to effectively silence the constitutive AF-1 activation domain and thereby repress estrogen-responsive target gene transcription. After estrogen stimulation, a ligand-induced conformational change within ERα could lead to enhanced affinity of the ERα for its cognate binding site and release of a BRCA1-containing repression complex, thereby liberating AF-1 and AF-2 to synergistically recruit coactivators and the RNA polymerase II holoenzyme to promote transcription (29). It is also possible that BRCA1 could function additionally as a barrier to the productive association of either unliganded and/or liganded ERα with promoter DNA, and this could underlie the previous observation that BRCA1 can inhibit ligand-dependent ERα transactivation (17, 18).

Interestingly, we observed that a deficiency of BRCA1 also leads to a reduction in the relative level of E2-mediated ERα activation. In both Brca1-nullizygous MEFs and BRCA1-deficient BG-1 (AS4) cells, the relative level of E2-mediated activation of a transfected ERα-responsive reporter gene was diminished when compared with Brca1-proficient cells. Furthermore, in AS4 cells, the endogenous estrogen-response genes that we monitored exhibited increased estrogen-independent expression and little or no estrogen-dependent stimulation when compared with BRCA1-proficient BG-1 (NEO1) cells. It is possible that the expression of these genes is largely derepressed in a BRCA1-deficient background and cannot therefore be increased substantially in response to estrogen.

Previously, Annab *et al.* (22) demonstrated that relative to parental or retroviral vector-infected BG-1 cell clones, BRCA1 antisense-infected BG-1 cell clones exhibit enhanced estrogen-independent growth in culture (22). Furthermore, BG-1 clone AS4, which exhibits severely reduced BRCA1 expression levels, exhibited increased tumorigenicity in ovariectomized nude mice compared with the retroviral vector-infected NEO1 cell clone (22). These observations suggest that forced reduction of

BRCA1 in BG-1 ovarian adenocarcinoma cells may influence estrogen-independent growth both *in vitro* and *in vivo*. Our observation that AS4 cells support significant increases in the estrogen-independent expression levels of different ERα-target genes compared with BRCA1-proficient NEO1 cells may provide a mechanistic basis for the estrogen-independent growth advantages that AS4 cells exhibit.

The finding that BRCA1 can function as a ligand-reversible barrier to transcriptional activation by unliganded ERα suggests the potential involvement of BRCA1 in the proliferative control of normal estrogen-regulated tissues. Thus, mutational inactivation of BRCA1 could result in persistent expression of estrogen-responsive genes in the absence of threshold levels of estrogenic stimulation. In this way, inappropriate hormonal responses brought about by BRCA1 mutation might possibly promote the proliferation of transformation-initiated cells.

Previous analyses have revealed that a significant proportion of BRCA1-associated breast tumors are negative for ERα expression (34). However, the loss of ERα expression in BRCA1-associated tumors is likely to represent a relatively late event in breast tumor progression, one that may have occurred after any proliferative advantages conferred upon transformation-initiated cells by homozygous BRCA1 mutation have ensued. Possibly, the down-regulation of ERα expression in BRCA1-mutated tumors could derive in part from negative feedback control enlisted by BRCA1-mutated breast epithelial cells to restrict the promiscuous expression of estrogen-responsive genes. Future studies should illuminate the mechanistic basis for BRCA1-mediated transcriptional repression of ERα and clarify its functional role in the larger network of hormone signaling pathways that control the growth, differentiation, and homeostasis of breast and ovary.

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BRCA1 and *BRCA2* in breast cancer

Wen-Hwa Lee, Thomas G Boyer

The inheritance of an autosomal dominant allele represents an identifiable predisposing factor in about 10% of all women with breast cancer. Most of these hereditary cases can be linked to germline mutations in either of two breast cancer susceptibility genes, *BRCA1* or *BRCA2*. Women who have a mutation in either of these genes have a cumulative lifetime risk of 60–80% and 20–40% for the development of breast and ovarian cancer, respectively. There is therefore a great need for new and effective measures for their management. Progress in our understanding of the normal biological function and regulation of *BRCA1* and *BRCA2* has shed new light on the molecular basis of hereditary breast cancer, and should provide a driving force for the development of diagnostic and therapeutic strategies.

BRCA1 and *BRCA2* are caretaker genes whose products function in the maintenance of global genome stability—ie, they ensure that the genetic integrity of a cell is not compromised by the unscheduled loss, duplication, or rearrangement of chromosomal DNA. A persistent threat to genome integrity is DNA damage arising from ongoing metabolic processes within the cell, as well as that elicited by extrinsic agents, including radiation and certain chemicals. Unrepaired or misrepaired DNA damage can compromise chromosomal stability, allowing a cell to escape normal restrictions on its growth.

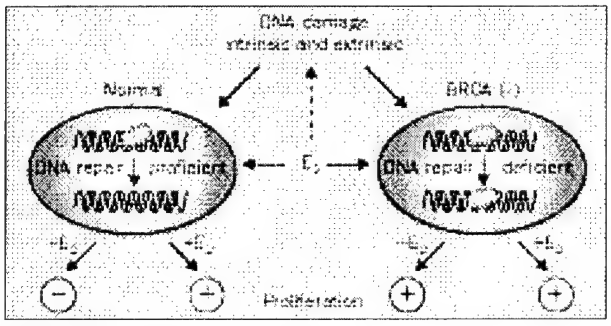
Genome integrity is ensured in part by a response system that has evolved to locate and effect the timely repair of damage to DNA. This response involves the assembly of DNA-repair protein complexes able to recognise and eliminate damage-induced lesions, and the synthesis of cell-cycle checkpoint control proteins that provide a sufficient window of opportunity to effect such repair. *BRCA1* and *BRCA2* occupy fundamental roles in coupling DNA damage-induced signals to downstream cellular responses, including damage repair and cell-cycle checkpoint activation.

Because the DNA damage-induced signalling pathways that converge on *BRCA1* and *BRCA2* are universally conserved, the genes are likely to function ubiquitously in the maintenance of genome integrity. Nonetheless, inactivation of *BRCA1* or *BRCA2* generally leads only to cancer of the breast or ovary. What then might constitute the molecular basis for the tissue-specific tumour suppressive properties of *BRCA1* and *BRCA2*?

The breast and the ovary are reproductive organs that rely on hormones, including oestrogen and progesterone, for growth, differentiation, and homeostastis. According to one theory, inactivation of *BRCA1* and *BRCA2* renders breast and ovary susceptible to tissue-specific effects of oestrogen-induced DNA damage. Thus, inactivating mutations in *BRCA1* and *BRCA2* could compromise the response of breast and ovarian epithelial cells to oestrogen-induced DNA damage, thereby resulting in inefficient or error-prone DNA repair. Global genomic instability and a concomitant accrual of functionally inactivating mutations within other genes involved in breast and ovarian tumourigenesis might then ensue. Alternatively, *BRCA1* might modulate hormone signalling pathways and control of cellular proliferation. *BRCA1* represses the transcriptional activity of the oestrogen and progesterone receptors, and mutational inactivation of the gene could, therefore, promote epithelial cell proliferation through altered expression of hormone-responsive genes.

These two models are not mutually exclusive and could suggest a combinatorial path to breast cancer, since they invoke *BRCA1*-mediated and *BRCA2*-mediated control at two distinct steps of tumourigenesis—initiation and progression. Thus, inappropriate expression of hormone-responsive genes could promote the proliferation of transformed cells arising through inefficient or error-prone repair of oestrogen-induced DNA damage. In this way, hereditary *BRCA1* and *BRCA2* mutations could render breast and ovarian epithelial cells particularly susceptible to tumourigenesis through perturbation of distinct hormone-dependent pathways (figure).

This knowledge could help to treat those carrying mutations in *BRCA1* and *BRCA2*, and might also be useful in the treatment of patients with sporadic, non-genetic breast cancers. Few mutations in *BRCA1* and *BRCA2* arise in sporadic breast cancers, suggesting that the perturbation of alternative pathways causes malignant disease in these cases. As caretakers of genomic integrity, *BRCA1* and *BRCA2* represent prime targets for therapeutic intervention—ie, targeted inactivation of *BRCA1*-specific and *BRCA2*-specific DNA-damage response pathways could render tumour cells sensitive to the genotoxic effects of radiation or chemotherapeutic agents, thereby offering the potential for improved combination therapies. In the last decade of the 20th century, *BRCA1* and *BRCA2* were identified and characterised. The role and regulation of their encoded products in DNA-damage response and repair, once identified, should expedite the design and implementation of strategies to delay, and ultimately to prevent, tumour formation.



Role of *BRCA* genes in breast cancer
E_2=oestrogen.

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Breast Cancer Susceptibility Genes

by Thomas G. Boyer and Wen-Hwa Lee

The last decade of the 20th century witnessed the identification and initial characterization of two major breast cancer susceptibility genes, *BRCA-1* and *BRCA-2*. Studies of the encoded BRCA proteins have revealed roles in the maintenance of chromosomal stability and in DNA damage response and repair, and studies continue to illuminate further biological activities. A greater appreciation of the involvement of *BRCA-1* and *2* in breast and ovary cells will increase the probability that recent advances in our understanding of their biological functions will be channeled effectively to the treatment and prevention of breast and ovarian cancer.

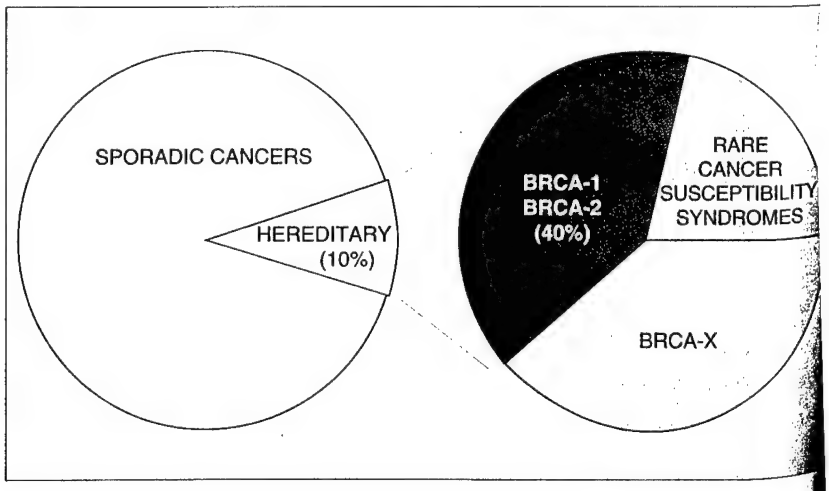
In the year 2002, approximately 200,000 American women will be diagnosed with breast cancer, the most common malignancy afflicting women in the United States. Among women who do not smoke, breast cancer is the primary cause of cancer-related death.

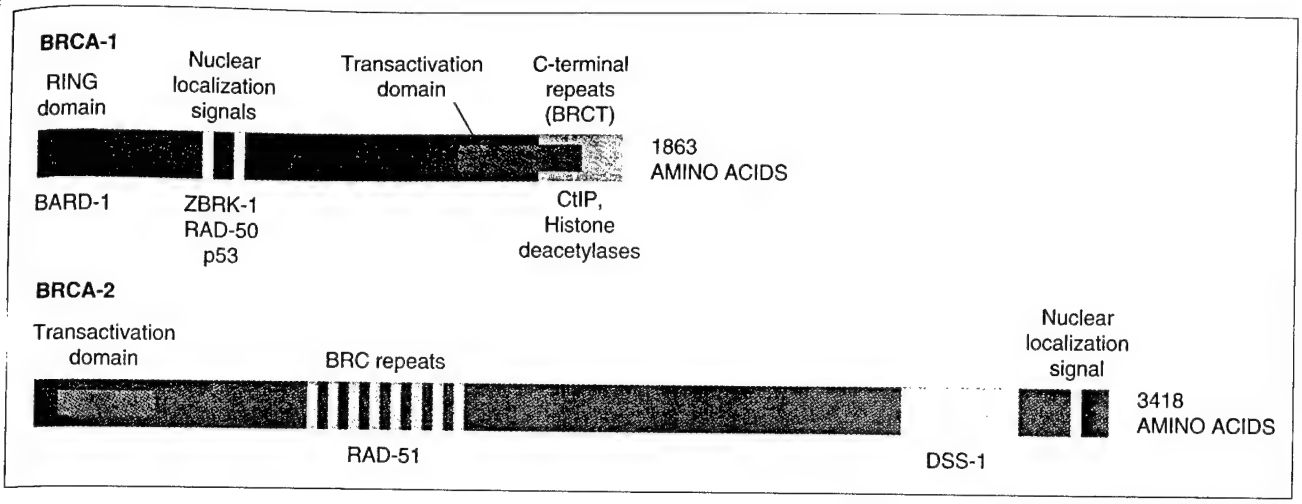
Although many factors influence a woman's lifetime risk for development of breast cancer, family history is one of the most powerful prognostic indicators. About 10% of all breast cancer cases can be linked to heritable transmission of an autosomal dominant allele. Thus a major achievement was substantiation that many of these hereditary cases could be linked to germline mutations in either of two breast cancer susceptibility genes, identified as *BRCA-1* and *2*.

Through linkage analysis of families affected by early-onset breast and ovarian cancer, *BRCA-1* was mapped to chromosome 17q21 in 1990 and cloned 4 years later. *BRCA-2* was mapped to chromosome 13q and cloned shortly thereafter. Mutations in *BRCA-1* are believed to account for 60 to 80% of hereditary breast and ovarian cancer cases and up to 20% of hereditary breast cancers only. *BRCA-2* mutations are linked to a similar percentage of inherited breast cancers, but in contrast to *BRCA-1*, they also predispose to male breast cancer.

Together, defects in these two genes account for about 40% of inherited breast cancers. Germline inactivation of one allele of either *BRCA-1* or *2* is sufficient to predis-

Mutations in BRCA genes account for almost half of hereditary breast cancers. The rest derive from mutations either in identified genes associated with rare cancer susceptibility syndromes or in unidentified susceptibility genes.





Structural and functional domains of BRCA-1 and 2 are named above each of the schematic proteins. Representative proteins that interact with BRCA-1 and 2 are identified beneath them.

pose a person to cancer, while cancer onset is invariably accompanied by loss of the remaining allele. Thus *BRCA-1* and *2* belong to the group of tumor susceptibility genes whose encoded products normally function to suppress tumor formation.

Mutations in other known tumor susceptibility genes, such as *p53*, the retinoblastoma gene *RB*, and the adenomatous polyposis gene *APC*, are found in both familial and sporadic tumors. Mutations in *BRCA-1* and *2*, however, are rarely detected in nonhereditary breast cancers, though it has been proposed that aberrant regulation of their expression or of the activity of their products could contribute to sporadic breast cancers.

Clearly, detailed knowledge of the normal biological functions of these proteins and of their regulation will be required for a thorough appreciation of how direct or indirect functional inactivation of *BRCA-1* and *2* leads ultimately to breast cancer. In this article, we begin with a description of the structural features of the BRCA proteins and then highlight recent insights into their biological role and regulation.

Protein Structures Are Clues to Functions

BRCA-1 is a nuclear phosphoprotein of 1863 amino acids characterized by the presence of a notable structural motif near each end.

At its amino terminus, BRCA-1 harbors a zinc-binding RING finger domain, which is a set of spatially conserved cysteine and histidine residues. More than 200 RING finger proteins of diverse function are potentially encoded by the human genome, so this domain is a relatively common structural motif. Recent studies have raised the possibility that the functional diversity apparent among RING finger proteins is tied to a common enzymatic activity.

The carboxy terminus of BRCA-1 includes tandem domains that are autonomous folding units defined by conserved clusters of hydrophobic amino acids. These are called BRCA-1 C-terminal or BRCT domains, and they have been found in other proteins implicated in DNA repair and cell cycle checkpoint control. No specific cellular function has so far been ascribed to the BRCT domain, but it is likely to be a protein interaction surface.

A third region in BRCA-1 also appears to be a functionally relevant protein interaction surface, but the structure of this region has not yet been defined. The same region includes two putative nuclear localization signals.

BRCA-2 is a nuclear protein of 3418 amino acids whose most prominent feature is eight tandem copies of a repetitive sequence termed the BRC repeat. Also notable is a region of about 500 amino

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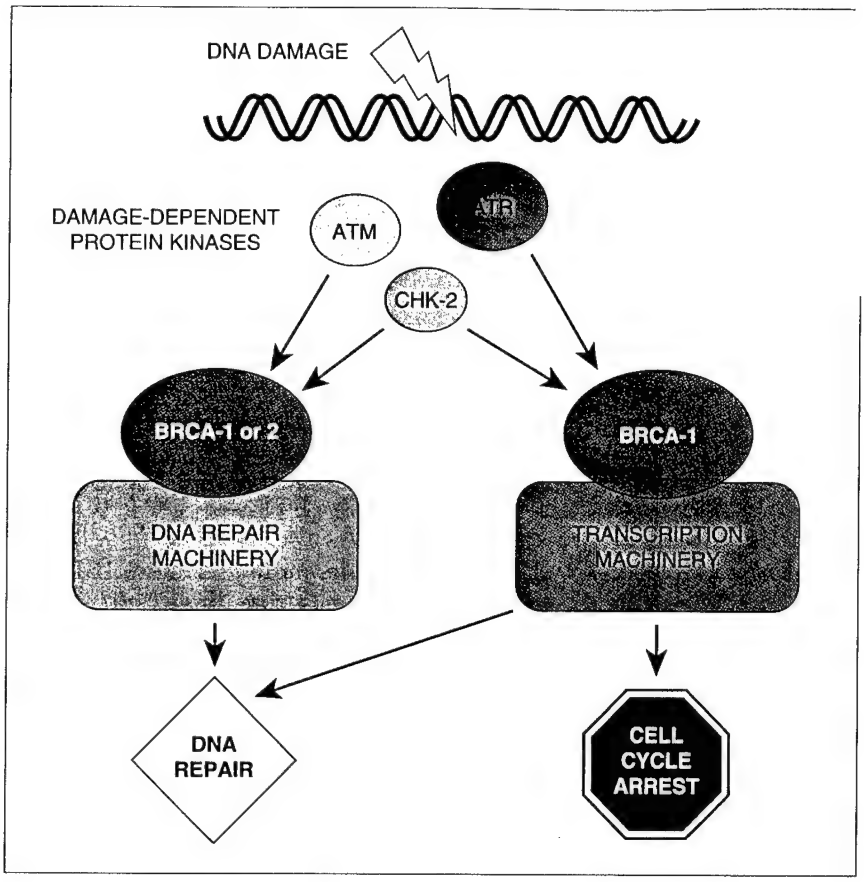


BRCA-1 and 2 couple signals of DNA damage to cellular responses, including damage repair and cell cycle checkpoint activities. Though much about this process is not yet understood, it is known that BRCA-1 is phosphorylated by any of several protein kinases, depending on the type of DNA damage. Both BRCA proteins interact physically with DNA repair proteins, and BRCA-1 also participates in transcription control of genes that encode DNA repair and cell cycle checkpoint control proteins.

acids near the carboxy terminus that is more highly conserved between human and mouse than the coding sequence as a whole.

BRCA-1 and 2 Are Caretakers of Genomic Stability

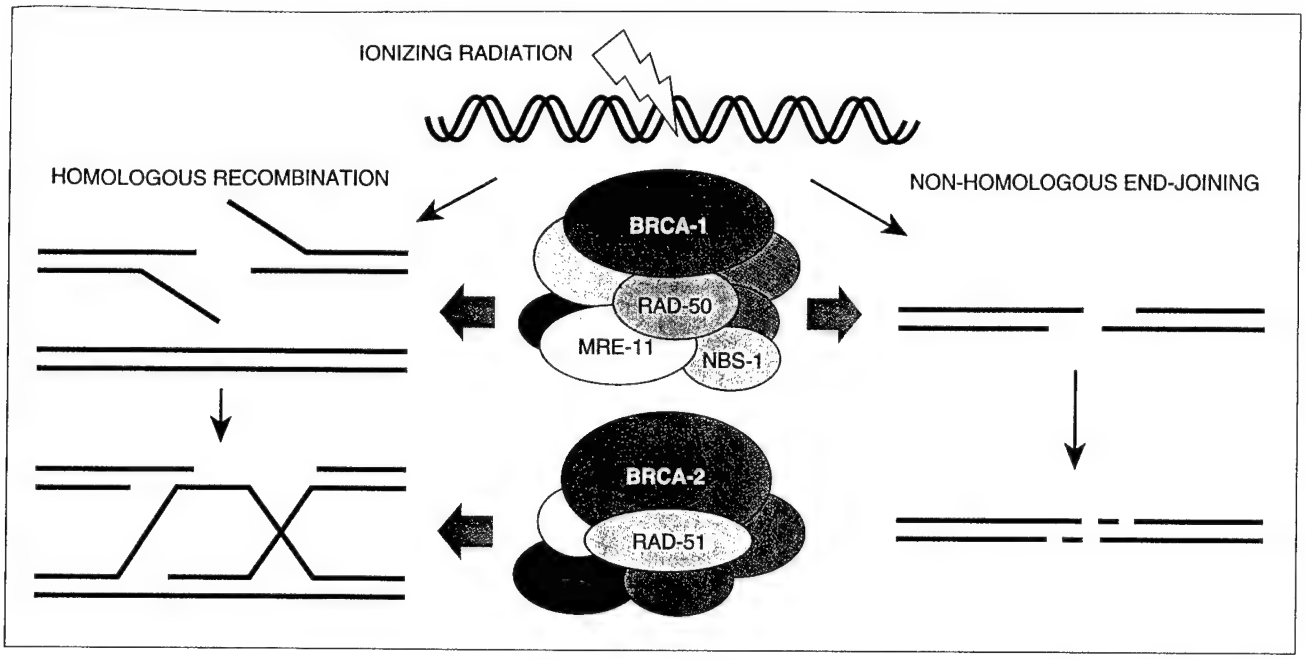
Insights into the biological functions of BRCA-1 and 2 have come from analyses of cells derived from BRCA-mutant human breast tumors and from embryos of mice carrying targeted deletions of the *BRCA* genes. Invariably, BRCA-deficient cells exhibit gross chromosomal abnormalities, typified by breaks, aberrant mitotic exchanges, and aneuploidy.

These sorts of DNA damage arising from ongoing metabolic processes within the cell or caused by extrinsic agents, including radiation and certain chemicals, are a persistent threat to genome integrity. A response system has evolved to locate damaged DNA and effect its timely repair. BRCA-1 and 2 are parts of that system, cellular caretakers ensuring that the genetic integrity of a cell is not compromised by the unscheduled loss, duplication, or rearrangement of chromosomal DNA.

The DNA damage response involves the assembly of protein complexes capable of recognizing and eliminating damage-induced lesions, as well as the synthesis of proteins that arrest cell cycle progression while the damage is repaired. Disruption of the damage response system can lead to replication or segregation of damaged chromosomal DNA, and that in turn can permit a cell to escape normal restrictions on its growth, which is practically the definition of cancer.

Evidence to implicate BRCA-1 and 2 in the DNA damage response has come from the observation that cells deficient in either protein are hypersensitive to a variety of DNA-damaging agents. A more specific function was suggested by the finding that cells

Discovery of the BRCA genes and early work on their protein products was described by Barbara Weber in the January/February 1996 issue of SCIENCE & MEDICINE.



deficient in either BRCA-1 or 2 exhibited overt defects in the repair of oxidative DNA damage. Further studies have documented direct interactions between BRCA-1 or BRCA-2 and individual protein components of the DNA repair machinery.

BRCA-1 has been linked to DNA repair through its interaction with a complex of three proteins, RAD-50/MRE-11/NBS-1, that operates in both nonhomologous and homologous recombinational repair of DNA double-strand breaks. The three-protein complex has been proposed to resect DNA ends at the sites of double-strand breaks in order to reveal sequence homologies through which recombination can ensue. What BRCA-1 does in its association with this complex remains to be established.

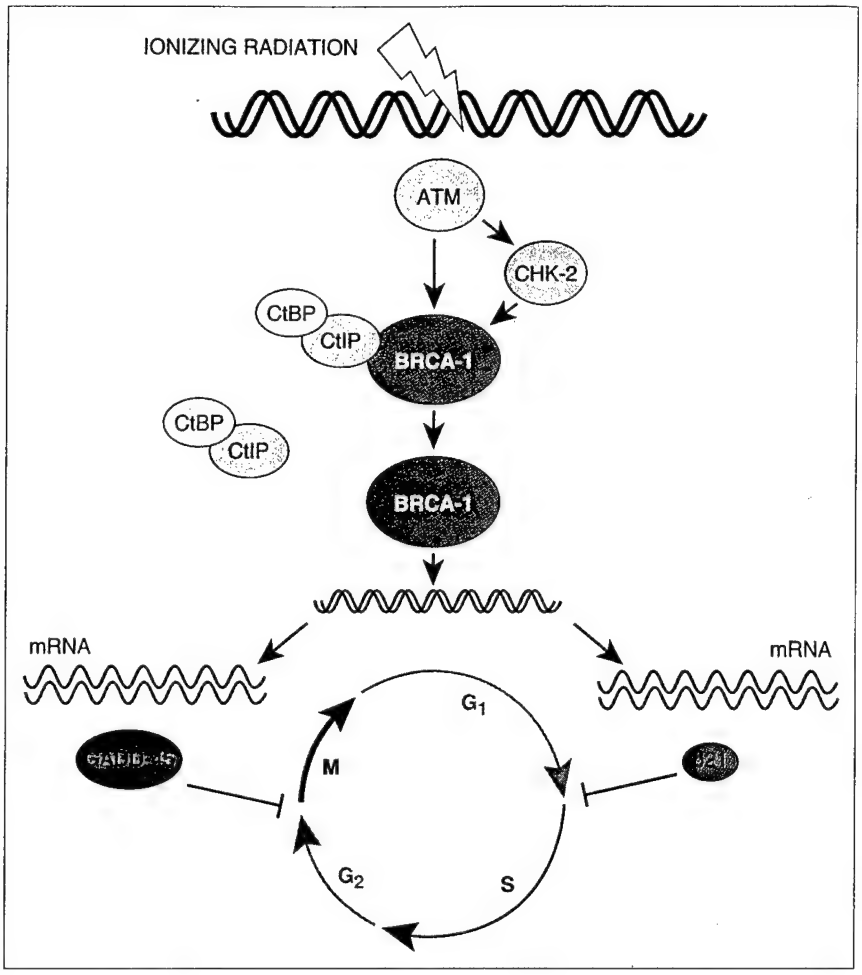
BRCA-1 is also a resident component of a large multiprotein complex that includes mismatch repair proteins. These and other proteins are involved in replication or in repair of DNA damage that can occur at replication forks. The association of BRCA-1 with these proteins suggests that it participates in resolving aberrant DNA structures that appear during replication or when replication is stalled.

A role for BRCA-2 in DNA damage repair has been suggested by the discovery that it interacts with a recombinase called, in mammals, RAD-51. Mammalian RAD-51 is a homologue of the prokaryotic RecA and yeast Rad51p proteins, the latter a member of the RAD-52 epistasis group. In yeast, RAD-52 epistasis proteins are required for repair of DNA double-strand breaks as well as mitotic and meiotic recombination.

Eukaryotic RAD-51 proteins, like RecA, have intrinsic ATP-dependent DNA binding activity. RAD-51 and single-strand DNA form a nucleoprotein filament that invades and pairs with a homologous DNA duplex, catalyzing homologous DNA pairing and strand exchange. Mouse embryos lacking BRCA-2 exhibit radiation hypersensitivity defects like those seen in mouse embryos lacking RAD-51.

The interaction between BRCA-2 and RAD-51 involves the BRC repeats in BRCA-2. Peptides corresponding to individual BRC repeats can inhibit multimerization of RAD-51 and block nucleoprotein filament formation. Whether this inhibitory activity is a physiological role for BRCA-2 in regulating RAD-51 activity has not been established.

DNA double-strand breaks induced for example by ionizing radiation are repaired by two processes in which the BRCA proteins are involved. In a complex with RAD-50, MRE-11, NBS-1, and other proteins, BRCA-1 takes part in both homologous recombination and nonhomologous end-joining. BRCA-2 complexed with RAD-51 is implicated in strand exchange during homologous recombination.



In association with co-repressors such as CtIP, BRCA-1 ordinarily represses transcription of cell cycle checkpoint control genes. In response to certain kinds of DNA damage, BRCA-1 and CtIP are phosphorylated by ATM. Phosphorylated CtIP dissociates from BRCA-1, leading to relief of BRCA-1-mediated transcriptional repression and consequent induction of *p21* and *GADD-45*. The protein products of those genes function in G_1-S and G_2-M cell cycle arrest, respectively.

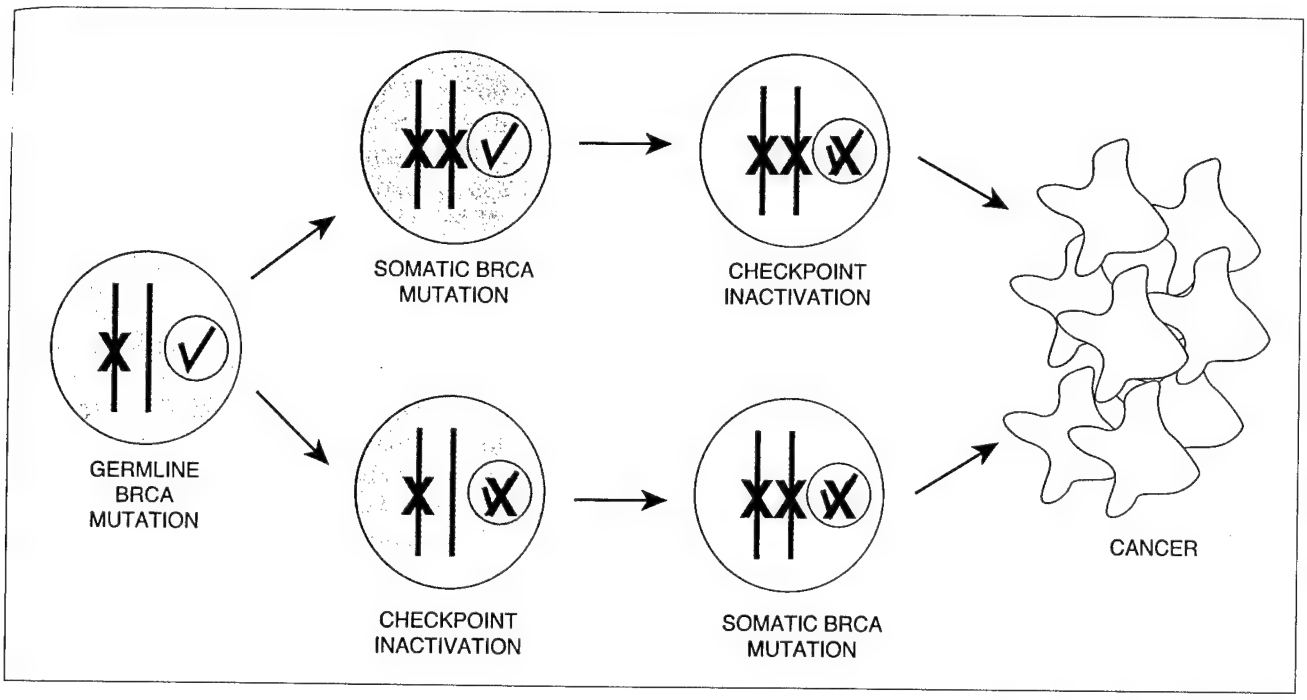
Consistent with that possibility, though, is the observation that formation of RAD-51 protein complexes, normally induced by DNA damage, is diminished in cells either deficient in BRCA-2 or in which the interaction between BRCA-2 and RAD-51 is specifically disrupted. It therefore seems clear that BRCA-2 is necessary for the assembly of RAD-51 complexes.

Processes of DNA repair must be coordinated with regulation of cell cycle transit so that damage is repaired before chromosomal DNA is replicated or segregated. There is considerable evidence that BRCA-1 occupies a central place in activation of cell cycle checkpoints when DNA damage is detected.

First, BRCA-1-mutant cells exhibit defects in DNA damage-induced S and G_2-M cell cycle checkpoints. Second, after DNA damage, BRCA-1 is rapidly phosphorylated by cell cycle checkpoint kinases, suggesting that it functions downstream of DNA damage sensors that trigger cell cycle checkpoints. And third, BRCA-1 has been shown to regulate expression of cell cycle checkpoint control genes, including *p21* and *GADD-45*, which function in G_1-S and G_2-M cell cycle checkpoints, respectively.

The role of BRCA-2 in cell cycle checkpoint control is much less clear. Where examined, DNA damage-induced cell cycle checkpoints appear to be largely intact in cells lacking wild-type BRCA-2. Indirect evidence exists to link BRCA-2 to G_2-M control, but it is not entirely clear whether this is an indirect effect secondary to the role of BRCA-2 in DNA damage repair.

The fact that specific disruption of the interaction between BRCA-2 and RAD-51 leads to loss of G_2-M checkpoint control suggests that this may be the case. Thus, BRCA-



2 inactivation could trigger existing checkpoints that monitor DNA structure, leading to delays in G_2-M progresson.

Collectively, the phenotypic characteristics of cells deficient in BRCA-1 or 2 suggest that these proteins are fundamental in the DNA damage response by participating in damage repair, cell cycle checkpoint control, or both.

Chromosomal instability arising from a defective DNA damage response has been proposed as the pathogenic basis for tumorigenesis accompanying BRCA deficiency. Paradoxically, chromosomal instability should lead to cell growth arrest or increased cell death, so the question is how BRCA-1 or 2 mutations might lead to the opposite effect.

One answer might lie in the observation that tumor cells deficient in BRCA-1 or 2 frequently harbor other inactivating mutations in cell cycle checkpoint control genes, including *p53*. Those mutations may circumvent the growth arrest that is normally induced by DNA damage and also inhibit p53-mediated apoptosis, permitting the survival of cells despite severe chromosomal damage.

On the other hand, inactivation of mitotic checkpoint genes could bypass mitotic arrest and permit aberrant chromosomes to segregate into progeny cells. This model is supported by experimental observations and suggests that the genetic instability arising in BRCA-1- or 2-deficient cells is pivotal in tumorigenesis, leading first to compensatory gene mutations that override damage-induced cell cycle arrest and apoptosis and subsequently to the accrual of functionally inactivating mutations of genetic loci involved in breast tumorigenesis.

BRCA-1 and 2 Regulate Cell Growth and Differentiation

Emerging evidence suggests important roles for BRCA-1 and 2 in the control of cell growth and differentiation. The clearest example is the observation that homozygous deletion of Brca-1 in mice results in early embryonic lethality accompanied by developmental retardation and cellular proliferation defects.

This outcome can be explained in part by the involvement of BRCA-1 and 2 in DNA repair,

Possible pathways to breast cancer in women carrying germline mutations in *BRCA-1* or *2*.

Somatic inactivation of the remaining allele gives rise to repair-deficient cells. Most of these cells ultimately die because of cumulative DNA damage and activation of cell cycle checkpoints. Rare repair-deficient cells (*green cells*) that survive can acquire additional mutations in cell cycle checkpoint control genes. These cells can survive in spite of genomic instability and give rise to tumors.

Alternatively, mutational inactivation of a cell cycle checkpoint gene may precede somatic inactivation of the remaining *BRCA* allele, resulting in repair deficient cells that can survive despite genomic instability. The result is the same.

because targeted deletions in *p53* or its downstream effector *p21* can rescue embryos with homozygous Brca-1 and 2 deficiency. Thus, cumulative DNA damage arising in the absence of Brca-1 and 2 has been hypothesized to trigger p53-mediated cell cycle arrest and apoptosis in the developing embryo, while inactivation of *p53* leads to cell cycle checkpoint bypass and survival.

However, inactivation of *p53* only partially rescues these embryos, which survive for only days longer in development. While the delayed embryonic lethality accompanying inactivation of *p53* has been ascribed to the accumulation of gross chromosomal defects that are incompatible with life, the possibility also exists that Brca-1 and 2 are required for transit through a critical point later in embryonic development.

Another line of evidence has come from studies of transgenic mice carrying a *Brca-1* allele that can be targeted for conditional inactivation specifically in the mammary glands of female mice. That inactivation elicits defects in ductal morphogenesis and also induces tumors that are associated with genetic instability, aneuploidy, and chromosomal rearrangements.

In addition to independently supporting a role for BRCA-1 as a breast tumor suppressor, this mouse model has revealed that BRCA-1 is critical in mammary epithelial development. Conditional inactivation of *BRCA-2* specifically in mammary gland has yet to be achieved, so the role of BRCA-2 in mammary gland formation remains to be established.

BRCA-1 and 2 Regulate Transcription

In parallel with the genetic studies, biochemical and molecular biological analyses have been carried out to determine how BRCA-1 and 2 execute their functions. The proteins have been linked to a variety of biological activities.

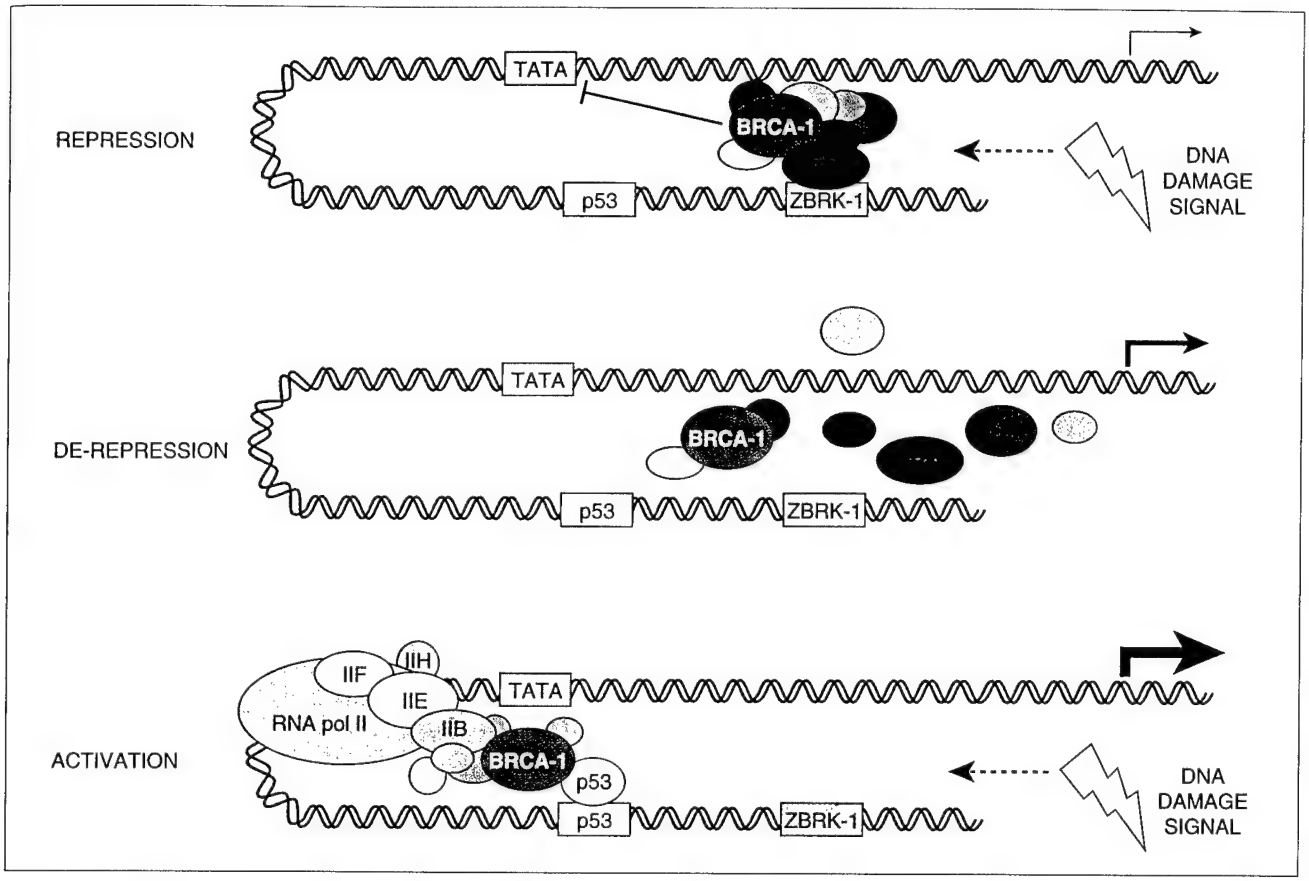
Involvement of BRCA-1 in transcriptional regulation was initially indicated by the identification near its carboxyl terminus of an acidic domain with an inherent transactivation function that is sensitive to cancer-predisposing mutations. This region interacts directly or indirectly with a variety of transcriptional co-activators, including the histone acetyltransferase p300 and hBRG-1, which is the catalytic subunit of a chromatin-remodeling complex called SW-1/SNF.

The same region, interestingly, also interacts with transcriptional co-repressors, including histone deacetylases and the CtIP/CtBP protein complex. *BRCA-1* mutations found in familial breast cancer compomise the trans-activation function but also abolish the binding of BRCA-1 to co-repressors. These observations have prompted the speculation that BRCA-1 may function like a nuclear receptor, either activating or repressing transcription depending on associated co-factors.

Gene expression profiling methods have disclosed that ectopic overexpression of BRCA-1 can induce or repress many genes implicated in cell cycle control, cell cycle regulation, and DNA replication and repair. By virtue of this transcriptional regulatory activity, BRCA-1 could influence cellular responses downstream of DNA damage signals, including DNA repair and cell cycle checkpoint activation.

BRCA-1-mediated regulation of *GADD-45* transcription illustrates how BRCA-1 might participate in cell cycle checkpoint control and also provides a model for how BRCA-1 can achieve gene-specific transcriptional regulation. *GADD-45* is a tumor suppressor gene induced by DNA damage. Its encoded product functions in G_2-M cell cycle checkpoint control.

Induction of *GADD-45* transcription in response to ultraviolet radiation and radiomimetic agents has been shown to depend on BRCA-1, and evidence exists to



suggest that the same may be true for ionizing radiation. It is also known that BRCA-1 interacts with a co-repressor, CtIP, to repress transcription of *GADD-45* and that this interaction is disrupted by DNA damage.

Neither BRCA-1 nor CtIP can bind DNA in a sequence-specific manner, however, so how these proteins are recruited to their target genes was an unresolved question. The answer was recently provided by identification of an intervening protein, named ZBRK-1, that binds to both BRCA-1 and a specific DNA sequence element present in a subset of BRCA-1's target genes, including *GADD-45*.

In this way, BRCA-1 can be physically tethered and functionally linked to specific regulatory loci. It is ZBRK-1 that actually represses transcription when it is bound to BRCA-1, so that BRCA-1 itself is a co-repressor. Potential ZBRK-1 binding sites have been identified in a larger group of genes inducible by DNA damage, so the ZBRK-1/BRCA-1 complex may be a global regulator of DNA damage-responsive genes.

A model has been proposed whereby ZBRK-1, BRCA-1, and CtIP coordinately repress a functionally diverse group of DNA damage-response genes in the absence of genotoxic insult, and that phosphorylation induced by DNA damage disrupts the network of interactions among these proteins, de-repressing transcription.

It must be emphasized that de-repression as an operative mechanism in transcriptional control of *GADD-45* and other inducible genes in vivo is likely to be coordinated with other mechanisms of gene activation. BRCA-1 has been reported to interact functionally with a variety of sequence-specific DNA-binding transcriptional activators, including the tumor suppressor p53.

In this regard, p53 appears to be an important link between BRCA-1 and transcriptional activation of DNA damage-inducible genes. It

Model for sequence-specific transcription control by BRCA-1 through its dual role as a co-repressor and a co-activator. ZBRK-1 is a transcriptional repressor that recruits BRCA-1 to its specific DNA binding sites in target genes, one of which is in intron 3 of *GADD-45*. BRCA-1 may then (1) recruit CtIP and CtBP to reorganize higher chromatin structure, (2) recruit histone deacetylase complexes to effect local gene silencing, or (3) interact with the basal transcription machinery.

In response to an appropriate DNA damage signal, BRCA-1-mediated repression of *GADD-45* transcription is relieved. That permits BRCA-1 to become a co-activator of, for example, p53, which also binds to intron 3 of *GADD-45*. Recent work has shown that BRCA-1 can also mediate transcriptional activation by activators that bind to the *GADD-45* gene promoter. BRCA-1 could mediate transcriptional activation by either (1) recruiting chromatin-modifying activities to facilitate transcription complex assembly at the promoter or (2) directly recruiting the RNA polymerase II holoenzyme to the promoter. In this model, damage-induced transcription of *GADD-45* results from concerted de-repression and activation.

lies at the heart of a cell-signaling pathway that is triggered by genotoxic stresses, including DNA damage. Stress-induced p53-initiated cell cycle arrest or apoptosis ensures the timely repair or elimination of potentially deleterious genetic lesions.

Significantly, p53 and BRCA-1 appear to regulate transcription of an overlapping set of DNA damage-inducible target genes, including *GADD-45*. This observation initially implied a functional interaction between these two important tumor suppressors, a prediction that has since been borne out experimentally.

BRCA-1 and p53 have been demonstrated to interact physically and to synergize functionally to activate transcription through a p53-binding site in a *GADD-45* intron. The ability of BRCA-1 to potentiate p53-dependent transcription without itself binding to DNA has led to the hypothesis that BRCA-1 functions as a p53-specific co-activator, possibly linking the biochemical activities of these two proteins to a common pathway of tumor suppression.

By being both a co-repressor and a co-activator of gene transcription, BRCA-1 appears to function as a link between parallel and perhaps synergistic pathways that lead to induction of DNA damage repair effectors. Before it can be understood how BRCA-1 integrates these dual functions, it will be necessary to decipher the mechanistic basis for its independent roles in activation and repression.

In contrast to BRCA-1, the part that BRCA-2 plays in transcriptional regulation is far less certain. Some evidence implicates BRCA-2 in transcription control, including, again, an inherent trans-activation function within the gene that is sensitive to cancer-predisposing mutations and an association with established transcriptional co-factors and histone acetyltransferases. However, the biological significance of these findings has not been demonstrated.

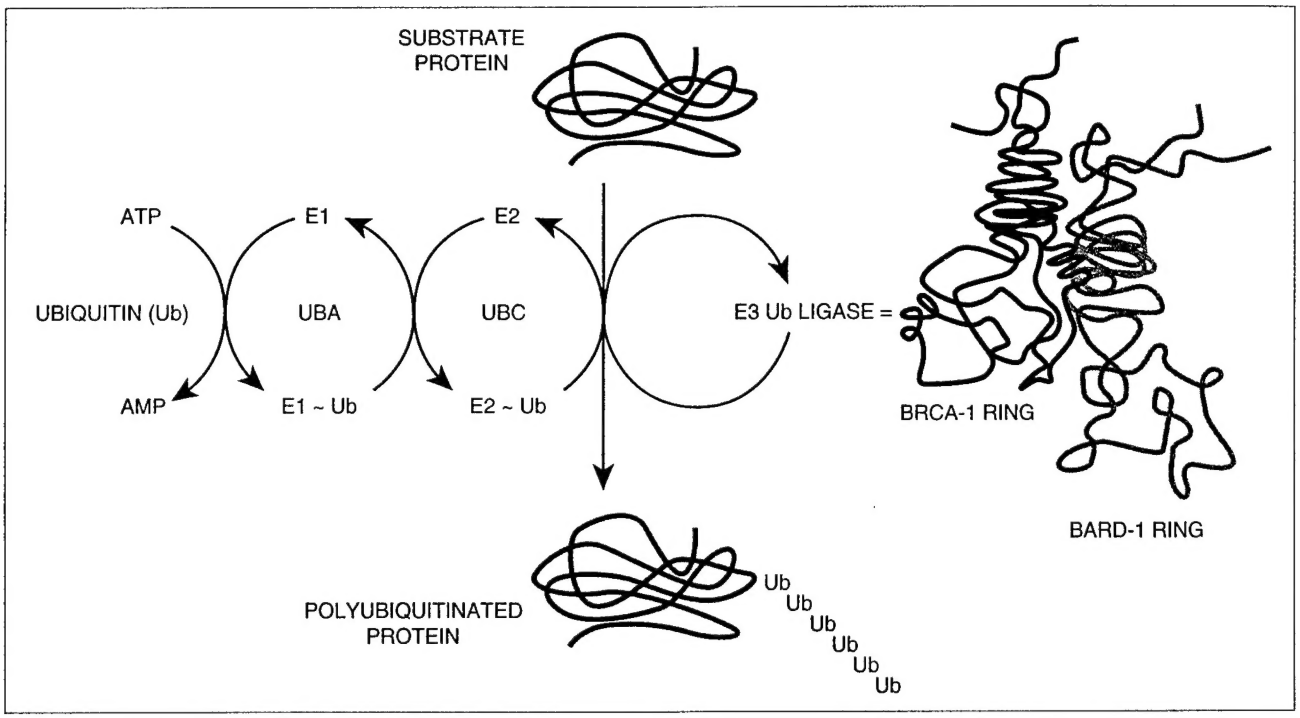
The mechanistic basis by which BRCA-1 participates in transcription control and DNA repair remains to be established. Most if not all of the cellular pool of BRCA-1 resides in stable complexes with other proteins, so one possibility is that BRCA-1 is a molecular scaffold that facilitates assembly of multiprotein machines.

Alternatively, the documented association of BRCA-1 with activities that modify chromatin could point to pleiotropic roles in DNA repair and gene transcription. BRCA-1 could variously promote or disrupt nucleosome-mediated condensation of DNA at gene promoters or DNA damage sites, thus precluding or facilitating access by transcription and repair factors, respectively.

Recent work has uncovered a ubiquitin ligase activity of BRCA-1, which raises the intriguing possibility that the protein's multiple functions could all derive from an ability to selectively mark proteins for destruction.

In the ubiquitination pathway, ubiquitin, a highly conserved 76-amino-acid protein, is covalently attached to lysine residues of proteins, targeting them for proteolysis. This binding generally requires the activities of E1 (UBA, ubiquitin-activating enzyme), E2 (UBC, ubiquitin-conjugating enzyme), and E3 (UBR, ubiquitin-recognition factor). The latter substance, also called ubiquitin protein ligase, is thought to confer substrate specificity. Together, these enzymes mediate the polyubiquitination of substrates, a signal that generally targets these proteins for proteolysis by the 26S proteosome.

By forming a heterodimer with another protein, BRCA-1 can function as an E3 ubiquitin ligase. Specifically, BRCA-1 interacts with a RING finger protein named BARD-1 through the respective RING domains of each protein. A heterodimeric complex formed by the isolated RING domains of the two proteins exhibits ubiquitin ligase activity in vitro.



Ubiquitin is a marker that tags other proteins for destruction. The sequence of events is shown here schematically.

A ubiquitin-activating enzyme E1 (UBA) is charged with ubiquitin, which is then transferred to a ubiquitin-conjugating enzyme E2 (UBC). A ubiquitin ligase E3 presumably functions as a platform for recruitment of both the E2 enzyme and a substrate protein, which is polyubiquitinated and thereby targeted for destruction.

A heterodimer formed by isolated RING domains of BRCA-1 and BARD-1 can function as an E3 ubiquitin ligase in vitro. Remaining surfaces on the two proteins could be involved in substrate recruitment in vivo. The structure of the heterodimer formed by the RING domains has been described.

Significantly, cancer-related missense mutations within the BRCA-1 RING finger abrogate this activity, suggesting that ubiquitin ligase activity may be important for the biological function of BRCA-1 in breast and ovarian tumor suppression. Presently, no physiological substrates of BRCA-1/BARD-1-targeted ubiquitination have been identified. But if BRCA-1 is involved in targeting proteins for ubiquitination, its participation in a wide range of cellular processes could be explained to some extent.

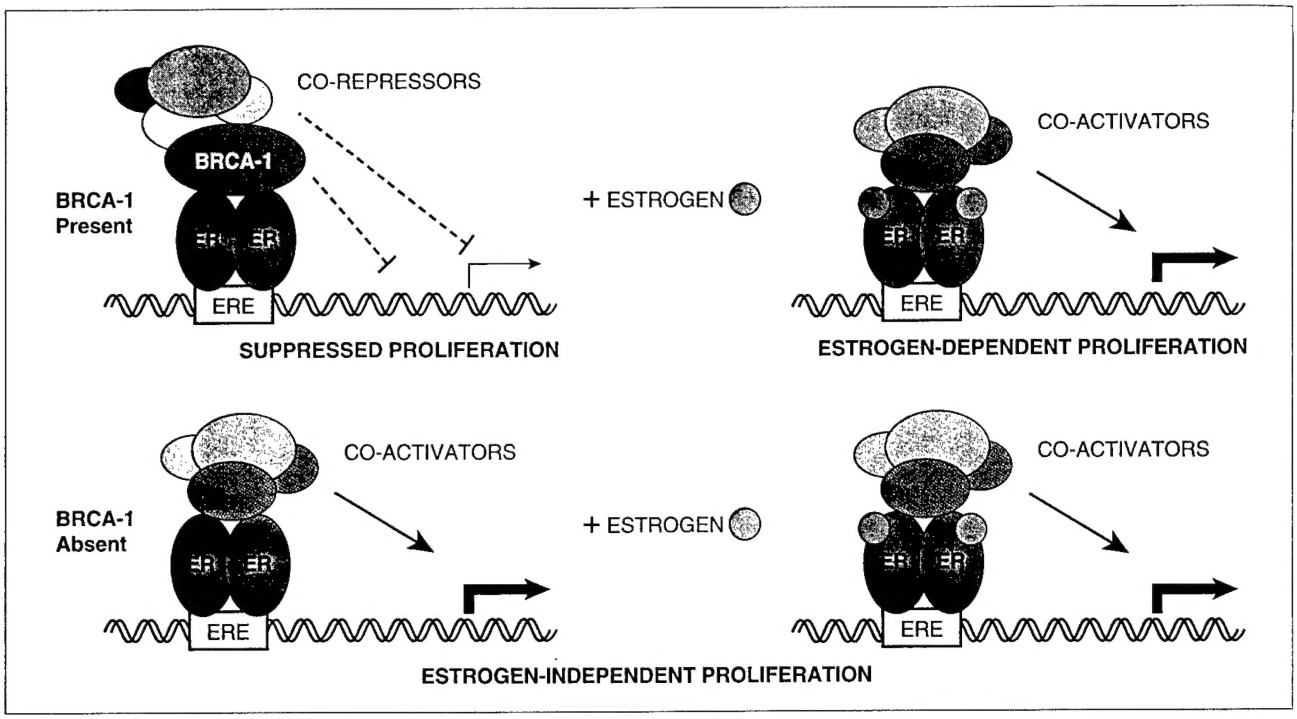
BARD-1 also interacts with a polyadenylation factor, CstF-50, which indirectly links BRCA-1 to RNA processing. Whether and how the ubiquitin ligase activity of BRCA-1 alone or in association with BARD-1 contributes to the functions of BRCA-1 is an important area for future investigation.

Tumor Susceptibility Is Tissue-Specific

DNA damage response pathways that converge on BRCA-1 and 2 are conserved across many cell types, so that BRCA-1 and 2 are likely to function widely in the maintenance of genomic integrity. Nonetheless, mutational inactivation of these genes leads principally to cancer of the breast and ovary. Why?

As reproductive organs, breast and ovary rely on hormones for growth and differentiation. At least two hypotheses invoking the action of hormones have been proposed to explain the tissue-restricted tumor suppressor functions of BRCA-1 and 2. According to one model, mutational inactivation of the *BRCA* genes renders breast susceptible to the tissue-specific effects of estrogen-induced DNA damage. A major oxidative metabolite of estrogen, 4-hydroxyestradiol, is genotoxic.

The suggestion is that inactivating mutations in *BRCA-1* or *2* could compromise the response of breast epithelial cells in particular to estrogen-induced DNA damage. Inefficient or error-prone DNA repair could then lead to genomic instability and a concomitant accrual of functionally inactivating mutations within other genes involved in breast tumorigenesis. Put another way, *BRCA-1* and *2* mutations might enhance the probability of tumor formation arising from estrogen-induced DNA damage.



BRCA-1 is a barrier to transcription of genes that are targets of the estrogen receptor (ER), possibly preventing cell proliferation by repressing unliganded ER bound to the estrogen response element (ERE). BRCA-1-mediated ER suppression additionally involves one or more co-repressors, minimally including a histone deacetylase activity.

In cells deficient in BRCA-1, ERE-bound ER is free to promote transcription of its target genes and cell proliferation independent of estrogen. Such transcription derives from recruitment of co-activators.

George Kuiper and colleagues discussed the two types of estrogen receptors in the July/August 1998 issue of SCIENCE & MEDICINE.

A second model proposes that BRCA-1 and 2 modulate hormone signaling pathways that induce cell proliferation. BRCA-1 has been shown to repress the transcriptional activity of the estrogen receptor (ER-α), so mutational inactivation of BRCA-1 could promote epithelial cell proliferation by altering expression of hormone-responsive genes.

The two models are not mutually exclusive and could suggest a combinatorial path to breast cancer, with BRCA-1-mediated control operating at two distinct steps in tumorigenesis, initiation and progression.

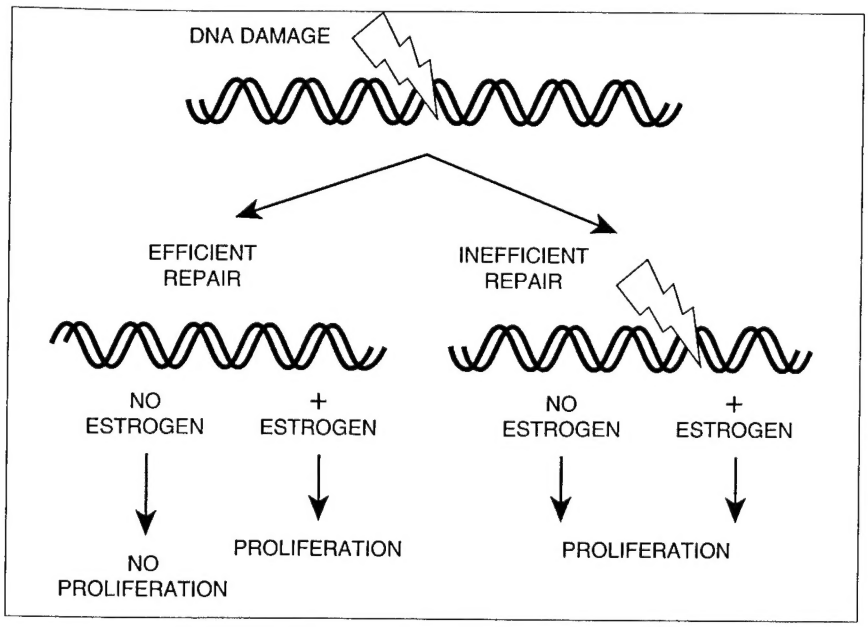
Models explaining how BRCA-1 acts through modulation of estrogen receptor function must account for the clinical observation that a significant proportion of BRCA-1-associated breast cancers are negative for ER-α expression. A definitive understanding of this phenomenon is precluded by the fact that it simply is not known how "ER-negative" tumors arise.

It has recently been shown that within the terminal ductal lobular unit, where breast cancers are believed to originate, there are at least three distinct epithelial cell populations: ER-α-positive cells that do not proliferative, ER-α-negative cells that do proliferate, and a small number of ER-α-positive cells that can proliferate as well.

Again, there are two principal models for the genesis of ER-α-negative epithelial-derived tumors, both of which are compatible with a role for BRCA-1 in the control of epithelial cell proliferation through functional interaction with ER-α.

In one model, ER-α-negative breast cancers arise from the loss of ER-α expression during the clinical evolution of cancers that were originally ER-α-positive. In this case, it is possible that the loss of ER-α expression is a relatively late event in breast tumor progression, one that may occur after any proliferative advantages conferred upon transformation-initiated cells by homozygous BRCA-1 mutation have ensued.

Alternatively, it has been proposed that ER-α-negative and ER-α-positive tumors are distinct entities that reflect the receptor status of their clonal origins. Recent data suggest a model in which proliferation of ER-α-negative cells is controlled by paracrine growth factors released from ER-α-positive cells in an estrogen-dependent manner.



Model for the role of the BRCA proteins in breast cancer. In normal breast epithelial cells, BRCA-1 and 2 ensure efficient DNA repair, thereby preserving genomic integrity in the face of genotoxic insult, including the action of estrogen metabolites. In addition, BRCA-1 restricts estrogen-independent expression of estrogen-responsive genes by directly inhibiting the unliganded estrogen receptor, thus rendering cells dependent on estrogen for growth. BRCA-deficient breast epithelial cells can develop unstable genomes through inefficient repair of damaged DNA and can become independent of estrogen for growth.

Here, mutational inactivation of BRCA-1 could promote growth factor-mediated proliferation of ER-α-negative tumors.

Finally, discovery of a second estrogen receptor subtype, ER-β, raises the possibility that this receptor mediates the proliferative response to estrogen in cells that are negative for ER-α expression. ER-β is expressed during the immortalization and transformation of ER-α-negative human breast epithelial cells in vitro.

The functional role of ER-β-mediated estrogen signaling pathways in the pathogenesis of breast cancer is currently unknown. However, the possibility exists that ER-β may also be subject to BRCA-1-mediated repression.

How might the knowledge now at hand concerning the biological functions of BRCA-1 and 2 be exploited to clinical advantage? For women genetically predisposed to *BRCA-1* and *2* mutations, restricted exposure to direct or indirect extrinsic sources of DNA damage might be warranted.

In reality, knowledge about BRCA-1 and 2 function might find its most useful applications in the treatment of the 90% of sporadic breast cancers for which no genetic linkage with an identifiable susceptibility locus can be found. In these sporadic cancers, pertubation of other pathways are likely involved in tumorigenesis. Nonetheless, as caretaker genes, BRCA-1 and 2 represent prime targets for therapeutic intervention.

For example, targeted inactivation of BRCA-1 and 2-specific DNA damage response pathways could render tumor cells particularly sensitive to the genotoxic effects of radiation or chemotherapeutic agents, offering the potential for improved combination therapies.

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